Clinicopathological and molecular characterization of colorectal micropapillary carcinoma

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Invasive micropapillary carcinoma is associated with frequent lymph node metastasis and adverse clinical outcome. Initially described as a variant of breast and ovarian carcinoma, it has subsequently been found in other organs, most recently the colon. Reports of colorectal micropapillary carcinoma to date are limited in number, and their molecular profile has not been established. The aims of the present study were to analyze their clinicopathological features and molecular profile, and compare them with those of conventional adenocarcinoma. Clinicopathological features of a cohort of 379 patients with primary colorectal cancer were retrospectively reviewed for the presence of the pattern characteristic of micropapillary carcinoma. We also assessed the expression of KRT7, KRT20, CEACAM5, MUC1 (EMA, clone E29), MUC1 (clone MA695), MLH1, MSH2, MSH6 and TP53 by immunohistochemistry. Genetic assessments of microsatellite instability, chromosomes 17p and 18q, and mutations in *TP53*, *BRAF* and *KRAS* were performed using DNA extracted from formalin-fixed, paraffin-embedded sections. In all, 60 of the reviewed cases (16%) had a micropapillary component that ranged from 5 to 95% of the tumor, characterized by a higher frequency of an infiltrative pattern, lymphovascular and perineural invasion, a higher depth of invasion and more positive lymph nodes than conventional adenocarcinoma. Immunohistochemistry for MUC1 (clone MA695) and MUC1 (EMA, clone E29) enhanced the characteristic inside-out staining pattern of the micropapillary carcinoma component, whereas the rest of the tumor showed luminal staining patterns. KRT7 expression was slightly increased in micropapillary carcinoma, but did not reach significance (17–3%, $P=0.1967$). The molecular parameters showed a higher frequency of *TP53* alterations and a low incidence of microsatellite instability and RER phenotype (loss of mismatch repair protein) in micropapillary carcinoma. With regard to the histological parameters, micropapillary carcinoma appears to be more aggressive than conventional colorectal adenocarcinoma. The molecular profile supports the hypothesis that micropapillary carcinoma carcinogenesis develops through the classical chromosomal instability pathway.

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Micropapillary carcinoma is accepted as an aggressive variant of colorectal adenocarcinoma, characterized histologically by small papillary cell clusters surrounded by lacunar spaces. It was described first in the breast and ovary, and subsequently in

other organs, including the bladder, lung, pancreas and salivary glands.^{1–7}

Previously published work found that the proportion of micropapillary carcinoma to the entire tumor ranged from 5 to 80% but was usually less than 30% of the entire lesion, with no pure cases of micropapillary carcinoma reported. Most cases were located in the sigmoid colon and the rectum.^{8–13}

Other studies have shown that micropapillary carcinoma is more commonly associated with lymphovascular invasion, lymph node metastasis

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and aggressive biological and clinical behavior. The inverted polarity of the cells that compose the micropapillary nests with an “inside-out” growth pattern, which has been shown by immunohistochemical and ultrastructural studies,^{14,15} is probably related to its high invasive potential.

Fewer than 130 cases of colorectal micropapillary carcinoma have been reported. The aim of the present study was to define the molecular profile of this variant, which has thus far not been well characterized because of a lack of clinical data.^{8–13}

Materials and methods

Patients, Tissues and Clinicopathological Features

A cohort of 379 cases of primary colorectal adenocarcinoma was retrieved from the archival files of Histopat Laboratories (Barcelona, Spain); cases were consecutive and collected between 1993 and 2008. Hematoxylin and eosin-stained sections (average four slides per tumor) were retrospectively reviewed by two pathologists to detect, quantify and localize a distinct micropapillary carcinoma pattern. We now characterize micropapillary carcinoma as neoplastic cell clusters with papillary morphology but without a true fibrovascular core, surrounded by empty lacunar spaces lined by strands of fibrocollagenous stroma. The tumor cells show eosinophilic cytoplasm, pleomorphic, hyperchromatic nuclei and a peculiar “inside-out” pattern of cell arrangement.^{1–7}

Clinicopathological features, summarized in Table 1, include the following parameters: gender and age of the patient, tumor location (proximal or distal to the splenic flexure), macroscopic type (ulcerated/stenosing or polypoid/exophytic), microscopic type (conventional adenocarcinomas or mucinous, when more than 50% of the tumor has an extra- or intracellular mucoid component)¹⁶ and histological grade defined by the ratio of glandular to solid pattern, following the WHO classification¹⁶ and categorizing mucinous adenocarcinoma as grade 3. The extent of tumor invasion and lymph node involvement was assigned according to the TNM system (sixth edition),¹⁷ and the presence or absence of metastasis and peritoneal invasion is reported. Clinical stage was classified based on Astler–Coller’s system.¹⁷ To classify the growth pattern, tumors were labeled as infiltrative when irregular penetrating or permeative growth was demonstrated on the periphery of the tumor, and as expansive when the tumor border was a smooth-pushing front.¹⁸ Cytological grading was divided into three categories: high, intermediate and low grade. Intramural and extramural thin-walled vessel invasion, venous vessel invasion and perineural invasion were also evaluated and recorded following the WHO classification.¹⁶ Thin-walled vessels are defined as vessels with only an endothelial cell layer in their walls, as opposed to venous

vessels that have a muscular wall. The presence of a Crohn-like lymphoid reaction was reported when at least three nodular aggregates of lymphocytes deep to the advancing margin of the tumor were found within a single low-magnification field ($\times 4$). The presence of tumor-infiltrating lymphocytes was characterized by four or more unequivocal intraepithelial lymphocytes in a single high-magnification field ($\times 40$) of an H&E-stained section, identified and counted in areas displaying the highest content of tumor-infiltrating lymphocytes.¹⁹ The tumor size was reported as the diameter maximum in millimeters. The distribution of different tumor patterns, including solid and mucinous, was reported in each case. The percentage of cribriform structures, consisting of a growing pattern of glands exhibiting small-sized secondary lumina and rounded in shape, was scored as an independent feature. Finally, the total number of lymph nodes detected and dissected was detailed.

The percentage of micropapillary component was recorded, and the case was classified as micropapillary carcinoma when it composed at least 5% of the tumor volume.

Immunohistochemistry and Molecular Studies

The immunohistochemistry profile included the expression of KRT7 (CK7), KRT20 (CK20), CEACAM5 (CEA), MUC1 (EMA, clone E29), and MUC1 (clone Ma695), and *TP53* and mismatch repair gene (*MMR*) expression. Molecular studies performed were *TP53* mutation, 17p and 18q losses, microsatellite instability status, *KRAS* mutation and *BRAF* V600E mutation; all summarized in Table 2.

Immunohistochemical Analyses

All immunohistochemistry analyses were performed following an avidin–biotin immunoperoxidase procedure. Immunohistochemistry was performed on 4- μ m-thick paraffin sections after rehydration. Endogenous peroxidase activity was inhibited with 0.1% hydrogen peroxide; deparaffinized sections were pretreated to induce epitope retrieval and were independently incubated with their corresponding monoclonal antibody. Secondary reagents were biotinylated horse anti-mouse antibodies (Vector Laboratories, Burlingame, CA, USA) and avidin–biotin complexes (Vectastain ABC PK 4000 ST, Vector Laboratories). A 3,3'-diaminobenzidine was used as the final chromogen and Harris-modified hematoxylin as the nuclear counterstain. Positive and negative controls—in which the primary antibody was omitted—were included in each experiment.

Immunohistochemistry studies for KRT7, KRT20, CEACAM5, MUC1 (EMA, clone E29) and MUC1 (clone Ma695) were performed in a subset of 60 cases. A total of 30 consecutive cases of

Table 1 Fisher's exact test/Wilcoxon signed-rank test of clinicopathological features and micropapillary component

<i>Categorical variable</i>	<i>% MC ≥ 5 (n = 60)</i>	<i>% MC < 5 (n = 319)</i>	<i>P-value</i>
<i>Gender</i>			0.6690
Male	37 (61.7)	185 (58.0)	
Female	23 (38.3)	134 (42.0)	
<i>Location</i>			0.6621
Proximal	24 (40.0)	113 (35.4)	
Distal	36 (60.0)	196 (61.4)	
Unknown	0	10 (3.1)	
<i>Configuration</i>			0.0236
Ulcerated/stenosing	42 (70.0)	171 (53.6)	
Polypoid/exophytic	18 (30.0)	145 (45.5)	
Unknown	0	3 (0.9)	
<i>Histology</i>			0.0829
Adenocarcinoma	57 (95.0)	275 (86.2)	
Mucinous	3 (5.0)	43 (13.5)	
<i>Grade (WHO)</i>			0.0018
1	23 (38.3)	171 (53.6)	
2	30 (50.0)	84 (26.3)	
3	7 (11.7)	64 (20.1)	
4	0	0	
<i>Extent of invasion (pT)</i>			0.0009
pT1	3 (5.0)	18 (5.6)	
pT2	2 (3.3)	51 (16.0)	
pT3	23 (38.3)	157 (49.2)	
pT4	32 (53.3)	93 (29.2)	
<i>Stage</i>			6.29×10^{-7}
1	3 (5.0)	59 (18.5)	
2	9 (15.0)	127 (39.8)	
3	38 (63.3)	108 (33.9)	
4	10 (16.7)	25 (7.8)	
<i>Lymph node involvement (pN)</i>			8.49×10^{-9}
0	12 (20.0)	192 (60.2)	
1	22 (36.7)	74 (23.2)	
2	26 (43.3)	53 (16.6)	
<i>Growth pattern</i>			6.92×10^{-9}
Infiltrative	58 (96.7)	199 (62.4)	
Expansive	2 (3.3)	118 (37.0)	
Unknown	0	2 (0.6)	
<i>Cytological grading</i>			0.0031
High grade	40 (67.0)	145 (45.5)	
Low or intermediate grade	20 (33.0)	172 (53.9)	
Unknown	0	1 (0.3)	
<i>Peritoneal invasion</i>			0.0012
Present	30 (50.0)	92 (27.6)	
Absent	30 (50.0)	231 (72.4)	
<i>Metastasis (pM)</i>			0.0479
Present	10 (16.7)	25 (7.8)	
Absent	50 (83.3)	294 (92.2)	
<i>Thin-walled vessel invasion</i>			7.47×10^{-12}
Present	51 (85.5)	120 (37.6)	
Absent	9 (15.0)	198 (62.1)	
Unknown	0	1 (0.3)	
<i>Venous vessel invasion</i>			1.86×10^{-5}
Present	28 (46.7)	61 (19.1)	
Absent	32 (53.3)	258 (80.9)	

Table 1 Continued

Categorical variable	% MC ≥ 5 (n = 60)		% MC < 5 (n = 319)		P-value
<i>Perineural invasion</i>					0.0006
Present	18 (30.0)		37 (11.6)		
Absent	42 (70.0)		282 (88.4)		
<i>Crohn-like lymphoid reaction</i>					0.0571
Present	15 (25.0)		122 (38.2)		
Absent	45 (75.0)		197 (61.8)		
<i>TIL</i>					0.5350
Present	6 (10.0)		44 (13.8)		
Absent	54 (90.0)		274 (85.9)		
Unknown	0		1 (0.3)		
<i>Adenomas</i>					0.4169
Present	18 (30.0)		78 (24.5)		
Absent	41 (68.3)		236 (74.0)		
Unknown	1 (1.7)		5 (1.6)		
Numerical variable	% MC ≥ 5 (n = 60) Mean $\pm$ s.d.	Median (IQ range)	% MC < 5 (n = 319) Mean $\pm$ s.d.	Median (IQ range)	P-value
Age (years)	65.8 $\pm$ 11.7	66.5 (16.0)	69.3 $\pm$ 12.0	71 (15.5)	0.0177
Tumor size (max. Ø, mm)	37.5 $\pm$ 12.3	40 (15.0)	43.8 $\pm$ 21.0	40 (25.0)	0.0429
Solid carcinoma (%)	11.5 $\pm$ 15.2	10 (16.25)	8.5 $\pm$ 19.2	0 (10.0)	0.0003
Mucinous carcinoma (%)	7.3 $\pm$ 15.3	0 (10.0)	13.1 $\pm$ 26.4	0 (10.0)	0.6345
Cribriform structures (%)	9.3 $\pm$ 18.5	0 (10.0)	8.1 $\pm$ 15.7	0 (10.0)	0.7652
Nodal involvement (n)	3.5 $\pm$ 3.3	2.5 (5.0)	1.9 $\pm$ 3.9	0 (2.0)	2.76 $\times 10^{-8}$

Abbreviations: MC, micropapillary component; TILs, tumor-infiltrating lymphocytes.
Bold values are statistical significance.

conventional adenocarcinoma, out of which 11 were located on proximal colon (37%) and 19 on distal colon (63%), and 30 cases of micropapillary carcinoma corresponding to the cases with more proportion of this component ($\geq 10\%$), out of which 14 were located on proximal colon (47%) and 16 on distal colon (53%). These analyses were carried out using primary mouse monoclonal antibodies, clones OV-TL12/30, Ks20.8, II-7 and E29 (Dako) and Ma695 (Novocastra), respectively. Immunohistochemistry evaluation was conducted using a double-blind method of scoring the presence or absence of expression in the tumor cells. Any proportion of positivity was considered.

MMR gene expression was assessed using the following primary monoclonal antibodies: anti-hMLH1 (clone G168-15), anti-hMSH2 (clone G219-1129) and anti-hMSH6 (clone 44) (BD Biosciences Pharmingen). In the tumor tissue, the absence of nuclear staining together with a positive internal control (normal mucosa or stromal cells, endothelial cells, lymphocytes) was considered as negative. Specimens with positive staining in a small cluster of neoplastic cells were considered to express the proteins.²⁰

Detection of TP53 protein was carried out using primary mouse monoclonal clone DO-7 (Dako). Immunohistochemistry semiquantitative double-blind

evaluation was conducted by scoring the estimated percentage of tumor cells showing nuclear staining.

Tissue Macrodissection and DNA Isolation

Ten 5- μ m-thick sections of formalin-fixed, paraffin-embedded tissues were used for each paired case (normal and tumoral samples) to perform manual scraping. DNA was isolated using a proteinase K-phenol/chloroform protocol.²¹ In each specific PCR reaction, 200 ng of DNA was used following assessment of DNA quality by PCR amplification of a 268-bp fragment of the *HBB* (human β -globin) gene,²² using a GeneAmp PCR System 9700 thermal cycler (PE Biosystems, Foster City, CA, USA).

Mutational Analyses

Amplicons 224 and 107 bp in size were obtained from single PCR reactions that spanned exon 15 of the *BRAF* gene and exon 1 of the *KRAS* gene. Direct sequencing of the purified amplicons was performed using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK). Exons 4–8 of the *TP53* gene were amplified in five independent PCR reactions. Mutational analysis was performed by SSCP in

Table 2 Fisher's exact/Wilcoxon signed-rank test of immunohistochemistry expression, molecular features and micropapillary component

<i>IHC expression</i>	% MC ≥ 5% (n = 30)	% MC < 5% (n = 30)	P-value
KRT7			0.1967
Positive	5 (16.7)	1 (3.3)	
Negative	225 (83.3)	29 (96.7)	
KRT20			1
Positive	29 (96.7)	28 (93.3)	
Negative	1 (3.3)	2 (6.7)	
CEACAM5.2			1
Positive	30 (100)	30 (100)	
Negative	0 (0)	0 (0)	
<i>Molecular variable</i>	% MC ≥ 5 (n = 60)	% MC < 5 (n = 319)	P-value
TP53 accumulation			0.1191
Present	39 (65.0)	169 (53.0)	
Absent	21 (35)	149 (46.7)	
Unknown	0	1 (0.3)	
TP53 mutation			0.0564
Present	45 (75.0)	193 (60.5)	
Absent	15 (25)	121 (37.9)	
Unknown	0	5 (1.6)	
TP53 altered (mutation and/or accumulation)			0.0343
Present	48 (80.0)	208 (65.2)	
Absent	12 (20.0)	108 (33.9)	
Unknown	0	3 (0.9)	
17p loss			0.4478
Present	27 (45.0)	141 (44.2)	
Absent	26 (43.3)	106 (33.2)	
Unknown	7 (1.2)	72 (22.6)	
18q loss			0.5409
Present	41 (68.3)	179 (56.1)	
Absent	17 (28.3)	93 (29.2)	
Unknown	2 (3.3)	47 (14.7)	
MSI (NCI panel)			0.0236
MSS	51 (85.0)	240 (75.2)	
MSI-L	3 (5.0)	21 (6.6)	
MSI-H	1 (1.7)	39 (12.2)	
Unknown	5 (8.3)	19 (6.0)	
MSI (NCI + 17p and 18q panel)			0.0123
MSS	49 (81.7)	215 (67.4)	
MSI-L	4 (6.7)	47 (14.7)	
MSI-H	1 (1.6)	34 (10.7)	
Unknown	6 (10.0)	23 (7.2)	
MMR loss of expression			0.0629
Present	1 (1.7)	27 (8.5)	
Absent	59 (98.3)	282 (88.4)	
Unknown	0	10 (3.1)	
RER phenotype (MMR loss and/or MIN)			0.0105
Present	1 (1.7)	40 (12.5)	
Absent	54 (90.0)	251 (78.7)	
Unknown	5 (8.3)	28 (8.8)	
KRAS mutation			0.4641
Present	27 (45.0)	121 (37.9)	
Absent	30 (50.0)	172 (53.9)	
Unknown	3 (5.0)	26 (8.2)	

Table 2 Continued

<i>Molecular variable</i>	% MC ≥ 5 (n = 60)	% MC < 5 (n = 319)	P-value
BRAF V600E mutation			0.1429
Present	9 (15.0)	26 (8.2)	
Absent	46 (76.7)	257 (80.6)	
Unknown	5 (8.3)	36 (11.3)	

Abbreviations: MC, micropapillary component; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, high microsatellite instability; MSI-L, low microsatellite instability; MSS, microsatellite stable.

Bold values are statistical significance.

polyacrylamide non-denaturing precast gels with two different electrophoresis conditions. Samples showing anomalous mobility were reamplified and their mutation confirmed by bidirectional sequencing.

Detection of Allelic Losses

Amplification of the TP53 CA dinucleotide repeat was performed for the analysis of allelic losses at chromosome 17p (*TP53* locus). Loss of heterozygosity (LOH) at the chromosome 18q region involving the *DCC* gene was analyzed using five microsatellite markers (D18S55, D18S58, D18S61, D18S64 and D18S69) by multiplex PCR. After PCR amplification, the fluorescent products were separated by capillary electrophoresis and analyzed using the GeneScan or GeneMapper software (PE Applied Biosystems). To calculate the LOH, peak heights of both alleles from normal and tumor samples were measured in relative fluorescent units. A LOH event was considered when the ratio $(N2 \times T1)/(N1 \times T2)$ was less than 0.5 or higher than 2.0. LOH was evaluated for each marker, and chromosome 18q allelic loss was considered when LOH was detected in at least one marker. Unstable and non-informative results for each marker were also recorded.

Analysis of Microsatellite Instability

Microsatellite instability status was evaluated using the five microsatellites from the NCI panel (BAT25, BAT26, D5S346, D2S123 and D17S250) in a multiplex PCR reaction.^{23–25} Fluorescent amplicons were analyzed on an automated ABI PRISM[®] 310 Genetic Analyzer using GeneScan or GeneMapper software (PE Applied Biosystems). Instability was assigned to a marker if its fragment pattern displayed either additional peaks or the appearance of separated novel fragments when the profiles of normal and tumor tissue were compared. Instability observed using the six microsatellites markers for elucidating the LOH status of chromosomes 18q or 17p (*TP53* locus) was also recorded, and a separate global microsatellite instability status was assigned with the 11 microsatellite markers.

In accordance with the consensus definitions of the US NCI, tumors were classified as exhibiting high microsatellite instability when 30% or more of the tested loci were unstable and as non-high microsatellite instability when less than 30% were unstable.

Statistical Analyses

Clinicopathological variables were investigated for their possible association with the presence of micropapillary pattern in at least 5% of the tumor. Categorical variables were analyzed by Fisher's exact tests of contingency tables, with odds ratio calculation as appropriate. Numerical variables were analyzed using the non-parametric Wilcoxon signed-rank test, which compares median differences. For all statistical tests, taking into account the exploratory character of our study, the *P*-values were considered as very strong evidence for *P*-values lesser than 0.001, a strong evidence for *P*-values between 0.001 and 0.01, and weak evidence for *P*-values between 0.01 and 0.05. Data were analyzed

using the R package v.2.3.1 (R Development Core Team, 2006) supplied with the exactRankTest package.^{26,27}

Results

Clinicopathological Findings

Table 1 summarizes the clinicopathological findings. Patients included 222 men and 157 women whose age ranged from 31 to 98 years (mean 68.7 ± 12.0 , median 70.0, IQ 17.0). Proximal location was seen in 137 tumors, and 232 tumors were located in the distal colon. The location of 10 tumors was not specified.

Microscopically, the micropapillary carcinoma pattern is composed of small irregular cell clusters surrounded by a thin lacunar space and separated by dense fibrous stroma (Figure 1a). Isolated infiltrating cells were also usually found. Tumor cells had a wide eosinophilic cytoplasm, a high-grade anaplasia (Figure 1b) and often showed a peculiar reverse cell polarity (Figure 1c). Immunohistochemistry for

Figure 1 Micropapillary pattern. (a) Characteristic cell clusters surrounded by lacunar spaces and dense fibrous stroma (H&E, $\times 100$). (b) MC cells displaying a high grade of anaplasia (H&E, $\times 400$). (c) Tumor nest with reverse cell polarity (H&E, $\times 400$).

Figure 2 (a) Immunohistochemistry for MUC-1 showing characteristic “inside-out” staining pattern in the MC component ($\times 400$). (b) The rest of the tumor shows luminal staining pattern (MUC-1, $\times 400$).

MUC1 (clone Ma695) in 85% of cases and MUC1 (EMA, clone E29) in 100% made this characteristic “inside-out” staining pattern in the micropapillary carcinoma component more evident (Figure 2a), whereas the rest of the tumor showed the classic luminal staining pattern (Figure 2b).

Micropapillary carcinoma involving at least 5% of the tumor volume was identified in 60 out of 379 colorectal carcinomas studied (16%) and was mainly located at the edge of the tumor. The proportion of this component varied, but in the majority of cases (95%), it represented less than 30% of the tumor. Out of 379 cases, 43 demonstrated between 5–10% micropapillary carcinoma pattern, 14 cases between 11–30% and only 3 cases more than 30%.

Comparison of clinicopathological features showed very strong evidence that pT ($P=0.00088$) and Astler–Coller stage ($P=6.29 \times 10^{-7}$) were higher in micropapillary colorectal carcinomas, grade ($P=0.0018$) was strongly related to the presence of micropapillary carcinoma, whereas mean age ($P=0.0177$) and tumor size ($P=0.0429$) were only weakly related. All tumors

Table 3 Distribution micropapillary component vs metastasis

% MC	Nodal metastasis (%) P-value	Distant metastasis (%) P-value
	8.49×10^{-9}	0.0479
< 5% ($n=319$)	127 (40)	25 (8)
≥ 5 –10% ($n=43$)	32 (74)	7 (16)
> 10–30% ($n=14$)	13 (93)	2 (14)
> 30 ($n=3$)	3 (100)	1 (33)

Abbreviation: MC, micropapillary component.

Bold values are statistical significance.

P-values reflect the different percentage of metastasis present in tumors with and without MC component.

Figure 3 Thin-walled vessel invasion (H&E, $\times 200$).

with a micropapillary carcinoma component displayed an infiltrative growth pattern and had more frequent nodal involvement ($P=8.49 \times 10^{-9}$; Table 3) and peritoneal invasion ($P=0.0012$), as well as a higher percentage of the solid pattern ($P=0.0003$). There was a very strong evidence that micropapillary carcinomas presented perineural ($P=0.0006$), venous vessel ($P=1.86 \times 10^{-5}$) and thin-walled vessel invasion ($P=7.47 \times 10^{-12}$; Figure 3). The micropapillary carcinoma group more commonly had a high cytological grade ($P=0.0031$) and distant metastasis ($P=0.0479$). There was weak evidence of a higher percentage of ulcerated/stenosing tumors in the micropapillary carcinoma group ($P=0.0236$). No significant differences were found with respect to sex of the patient, location, presence of adenomas, tumor-infiltrating lymphocytes, Crohn-like peritumoral reaction or the percentage of mucinous or cribriform patterns.

Immunohistochemistry and Molecular Findings

KRT20 staining was positive in nearly all micropapillary carcinoma cases (97%) and in the majority (93%) of conventional adenocarcinomas. No statistical differences were found between the groups

($P=1.00$). Out of 30 conventional adenocarcinomas studied by KRT7, only 1 case of proximal location showed KRT7 expression, whereas 5 out of 30 micropapillary carcinoma cases were positive, 2 cases of proximal location and 3 cases of distal location. KRT7 expression was more frequent in micropapillary carcinoma than in conventional adenocarcinoma (17 vs 3%), but this difference did not reach statistical significance ($P=0.1967$). KRT7 expression was often located at the peripheral areas of the tumor, corresponding to the transition areas from glandular to micropapillary carcinoma pattern. Finally, CEACAM5 expression was observed in all of the micropapillary carcinoma and conventional adenocarcinomas (Table 2).

The molecular findings are also summarized in Table 2. The difference in the expression of *TP53* and *MMR* genes by immunohistochemistry did not reach significance. With weak evidence, a higher proportion of *TP53* alterations (mutation and/or accumulation) was detected in the micropapillary carcinoma group ($P=0.0343$). However, no differences in the presence of *KRAS* or *BRAF* mutations were observed between carcinomas with and without micropapillary pattern, nor were differences found in the losses of 17p or 18q between the groups. There was a weak statistical evidence in the microsatellite instability statuses of these two groups of carcinomas using either the NCI microsatellite panel ($P=0.0236$) or the panel with 11 microsatellites ($P=0.0123$), with high microsatellite instability classification being rare among micropapillary carcinoma cases.

Discussion

The histological micropapillary carcinoma profile described in other studies^{8–13} was characterized by small clusters of tumor cells with a clear lacunar space around the tumor nest (Figure 1a). The nuclei were moderately to highly pleomorphic, and the cytoplasm was abundant and showed eosinophilia (Figure 1b). The micropapillary focus was often associated with single cell infiltration. Many tumor nests had a peculiar reverse glandular polarity (Figure 1c), which was also confirmed immunohistochemically by the membranous staining pattern of MUC1 (clone MA695), toward the stromal pole only, in the tumor cell clusters (Figure 2a).

Micropapillary pattern has overlapping morphological features, with the histological appearance of the tumor budding in the front of invasion of many colorectal carcinomas. However, the later, which reflects the epithelium–mesenchymal transition at the edge of invasion, lacks the characteristic “inside–out” staining pattern for MUC1 observed in the micropapillary carcinoma.²⁸

MUC1, a glycoprotein typically located in the apical cell surface of normal glandular epithelium, is thought to have an important role in lumen

formation and generally inhibit interaction between the cell and the stroma.^{8,14} Therefore, MUC1 interference with intercellular adherence is a key factor in the luminal formation of normal glands and probably also in the detachment of cells from the stroma. In invasive micropapillary carcinoma, the apical surface of inverted tumor cells faces the stroma and produces MUC-1, which directly interacts with the stromal cells. This process could have an important role in determining the characteristic morphological features of micropapillary carcinoma (lacunar space) and its invasive potential and aggressive behavior.^{8,14,15}

The aggressive profile of tumors with the micropapillary carcinoma component was highlighted in a study carried out by Haupt *et al*.¹⁰ These authors found a major frequency of lymph node metastasis ($P=0.001$), lymphovascular invasion ($P<0.050$) and distant metastasis ($P=0.311$). Kim *et al*⁹ found an association between the presence of the micropapillary carcinoma component and lymphovascular invasion ($P=0.011$), nodal metastasis ($P<0.001$), major number of lymph nodes affected ($P=0.006$), advanced stage ($P<0.001$) and major frequency of distant metastasis ($P<0.001$). In agreement with these two reports,^{9,10} we found a significantly greater proportion of cases with lymphovascular invasion and a higher number of affected lymph nodes. Furthermore, we also demonstrated a highly significant association between the presence of micropapillary carcinoma and the extent of invasion, with most micropapillary carcinoma cases (92%) classified as either T3 or T4. The grade and overall stage were also significantly higher in the micropapillary carcinoma group. In addition, vascular (thin-walled and venous vessel) and perineural invasions were significantly more frequent in carcinomas with the micropapillary carcinoma component.

The incidence of micropapillary carcinoma in the literature ranges from 9 to 19%, and the proportion of the micropapillary carcinoma component with respect the whole tumor is also variable but usually less than 30% of the tumor volume and mainly located at the periphery of the tumor.^{9,10}

Here, we present an analysis of a series of 379 colorectal carcinomas, including 60 cases with at least 5% micropapillary component (16%). Most of our micropapillary carcinoma cases (95%) contained between 5 and 30% of the micropapillary carcinoma component, and no tumors were pure micropapillary carcinoma. These proportions are very similar to those obtained by Kim *et al*⁹ and Haupt *et al*.¹⁰

The low proportion of micropapillary carcinoma component in the global tumor volume and its prognostic relevance make the number of sections studied crucial to keep in mind. An increase in the number of sections routinely studied, especially sampling the tumor-infiltrating edge, can likely result in a higher incidence of detection of the colorectal micropapillary carcinoma variant. In

colorectal biopsies, as in the surgical specimens, it is very important to always report the presence of micropapillary carcinoma, however, small its proportion in the tumor, because the bad prognosis does not depend on this ratio, but rather on the sole presence of this component (Table 3).

In polypectomy specimens having adenoma with malignant transformation, the finding of a micropapillary component should also be emphasized as an indicator of more aggressive potential and a high probability of regional lymph node metastasis. In these situations, we agree with Sonoo *et al* who recommend surgery as a more suitable procedure.²⁹

With regard to the immunohistochemistry profile, Kim *et al*⁹ do not report significant differences between colorectal carcinomas with or without a micropapillary carcinoma component. Nearly all cases of both micropapillary carcinoma and conventional adenocarcinoma showed immunoreactivity for KRT20 (95–100%), whereas KRT7 staining was usually negative (80–95%). In our immunohistochemistry study, a remarkable incidence of KRT7 expression (17%) was observed in the micropapillary carcinoma component, even when the subset of cases studied was slightly unbalanced to distal location. This finding could be significant when studying metastatic adenocarcinomas of uncertain origin with a micropapillary pattern. KRT20 expression is in agreement with colorectal carcinoma, ruling out other origins such as breast, lung or ovarian serous carcinoma.^{4,30,31} KRT7 immunoreactivity does not rule out possible colorectal origin. The expression of both KRT7 and KRT20 can suggest an origin in the pancreas or urothelium, but again do not exclude colorectal carcinoma.

High microsatellite instability in colorectal carcinoma has been associated with a good prognosis, and typically is associated with an expansive growth pattern and absence of *TP53* alterations. In our series, 58 out of 60 cases of micropapillary carcinoma had an infiltrative growth pattern and a higher proportion of *TP53* alterations. The observed differences in the presence of *TP53* mutations analyzed separately did not reach significance, although significance may be obtained with more extensive study. On the other hand, *TP53* accumulations clearly showed no differences between carcinomas with and without a micropapillary pattern, but it is well known that there is not always a direct association between *TP53* accumulation and presence of *TP53* gene mutations. Mutations that generate stop codons or frameshifts result in the absence of detectable protein expression, whereas *TP53* accumulation can also be due to alterations in other proteins involved in the *TP53* pathway.^{32,33}

When comparing the molecular profile of micropapillary carcinoma and conventional colorectal adenocarcinomas, although it did not reach a strong statistical evidence, microsatellite instability status was significantly different from direct assessment

with two microsatellite panels (NCI or NCI + 17p + 18q), and significant differences were also detected when considering, as a whole, MMR loss and/or microsatellite instability status (RER phenotype). These data constitute a relevant finding of our molecular study because they demonstrate an association between the presence of the micropapillary carcinoma phenotype and the classical chromosomal instability pathogenic pathway and, consequently, a worse prognosis.

In conclusion, colorectal micropapillary carcinoma appears to be more aggressive than conventional colorectal adenocarcinoma, regardless of its proportion. Thus, we must highlight the importance of carrying out exhaustive research into the presence of this component in the pathological study of colorectal cancer.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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