CLINICOPATHOLOGICAL AND MOLECULAR CHARACTERISATION OF COLORECTAL MICROPAPILLARY CARCINOMA.

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INTRODUCTION

Micropapillary carcinoma (MC) is a distinctive pattern of carcinoma characterized by small papillary cell clusters surrounded by lacunar spaces. It has been reported in ovary, breast, urinary bladder, ureter, lung, parotid gland and recently in colon and is associated with frequent lymphovascular invasion, lymph node metastases and poor clinical outcome.

The micropapillary component constitutes typically no more than 30% of the tumor volume coexisting with conventional carcinoma. It has been mostly described at the invasive edge of the tumor and has also been observed in metastatic foci.

The morphological and molecular profile of this type of colonic carcinoma has not yet been well studied since, to the best of our knowledge, there are only two series of colorectal micropapillary carcinoma published.

PATIENTS AND TISSUES

Hematoxylin and eosin-stained sections of a cohort of 337 consecutive cases of primary colorectal adenocarcinoma were retrospectively reviewed by two pathologists (average 4 slides per each tumor). Clinicopathological features were evaluated according to previously reported criteria and are summarized in table 1. The percentage of micropapillary component was recorded and the case was classified as MC when involved at least 10% of the tumor volume.

METHODS

Tissue Macrodissection and DNA Isolation

Ten 5µm thick sections were used for each paired case (N and T samples) to perform manual scrapping. DNA was isolated using a proteinase K-phenol/chloroform protocol. Two hundred ng of DNA were used for each specific PCR reaction after the assessment of DNA quality by PCR amplification of a 268bp fragment of the human ß-globin gene.

Immunohistochemistry

All immunohistochemical analyses were performed following an avidin-biotin immuno-peroxidase procedure. Positive and negative controls were included in each experiment. MissMatch Repair gene (MMR) expression was performed using the following primary monoclonal antibodies, anti-hMLH1 (clone G168-15), anti-hMSH2 (clone G219-1129), and anti-hMSH6 (clone 44) (BD Biosciences Pharmingen). Lack of protein expression was recorded when none of the tumor nuclei stained for hMLH1, hMSH2 or hMSH6. Positive staining of nuclei in intact adjacent crypt bases and lymphocytes served as an internal control. Tumors with intact expression in any tumor nuclei were recorded to have normal protein expression.

Detection of p53 and Ki-67 proteins was carried out using primary mouse monoclonal clones DO-7 and MIB-1 respectively (DAKO). Immunohistochemical evaluation was conducted double-blind by scoring the estimated percentage of tumor cells showing nuclear staining. In cases with p53 and Ki-67 intratumoral heterogeneity the score was produced by estimating the percentage of positive cells in microscopic fields displaying the most intense immunoreactive tumor cells.

Mutational Analyses

Amplicons of 224 and 107bp 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 to 8 of the TP53 gene were amplified in five independent PCR reactions. Mutational analysis was performed by SSCP in polyacrylamide non-denaturing pre-cast gels at two different electrophoresis conditions. Samples showing anomalous mobility were reamplified and their mutation confirmed by bidirectional sequencing.

Detection of Allelic Losses (LOH)

Amplification of the P53CA dinucleotide repeat was performed for the analysis of allelic losses at 17p chromosome (TP53 locus). LOH at 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 analysed using the GeneScan 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 (N2xT1)/(N1xT2) was less than 0.5 or higher than 2.0. LOH was evaluated for each marker, and chromosome 18q allelic loss was considered when loss of heterozygosity was detected in at least one marker. Unstable and non informative results for each marker were also recorded.

Analysis of Microsatellite Instability (MSI)

MSI status was evaluated using the five microsatellites from the NCI panel (BAT25, BAT26, D5S346, D2S123 and D17S250) in a multiplex PCR reaction. Fluorescent amplicons were analyzed on an automated ABI PRISM® 310 Genetic Analyzer using the GeneScan 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 with each other. In addition instability observed using the six microsatellites markers aimed at elucidating the LOH status of chromosomes 18q or 17p (TP53 locus) was also recorded and a separate global MSI status was assigned with the 11 microsatellite markers.

According to the consensus definitions of the US NCI, tumors were classified as exhibiting high microsatellite instability (MSI-H) when 30% or more of the tested loci resulted unstable and non-MSI-H when they were less than 30%.

Statistical Analyses

Clinicopathological variables were investigated for their possible association with the presence of micropapillary pattern in at least 10 % of the tumor. Categorical variables were analyzed by Fisher's exact tests of contingency tables with odd ratio (OR) calculation as appropriate. Numerical variables were analyzed using the nonparametric Wilcoxon signed rank test that compares median differences. For all the statistical tests, probability values (*p*-values) were considered significant when less than 0.05. Data were analyzed using the R package v.2.2.0 (©2005, R Development Core Team) supplied with the exactRankTest package.

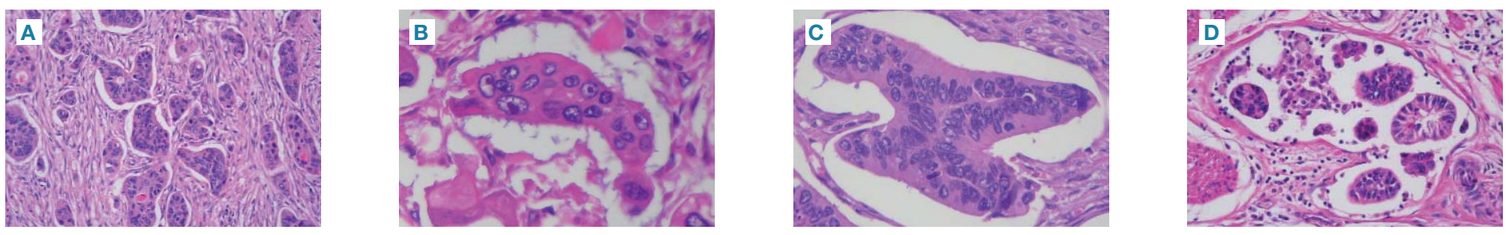

Figure 1. Histologic findings of micropapillary pattern. A, characteristic cell clusters surrounded by lacunar spaces and dense fibrous stroma (H&E: 100x). B, MC cell displaying a high grade of anaplasia (H&E: 400x). C, tumor nest with reverse cell polarity (H&E: 400x). D, thin-walled vessel invasion (H&E, 200x).

RESULTS

Clinicopathological Findings

Table 1 summarizes clinicopathological findings. Briefly, micropapillary component, involving at least 10% of the tumor volume, was identified in 27 of 337 cases (8%). The characteristic small irregular cell clusters were surrounded by a lacunar space and separated by dense fibrous stroma (figure 1A). They were typically found along with images of infiltrating isolated cells. The cells that constitute these cell clusters have a wide eosinophilic cytoplasm, a high grade anaplasia (figure 1B) and often present a reversal in cell polarity (figure 1C). Comparison of clinicopathologic features showed that the grade (p=0.017), pT and clinical stage were significantly higher in MC colorectal carcinomas (p<0.001). All tumors with MC component displayed an infiltrative growth pattern, had more frequent nodal involvement, peritoneal invasion and a higher percentage of solid pattern (p<0.001). There were also a significantly greater number of MC carcinomas presenting perineural, venous vessel and thin-walled vessel invasion (p<0.001) (figure 1D). The MC group more often had high cytologic grade (p=0.027) and distant metastasis (p=0.047). There was a higher percentage of ulcerated/stenosing tumors in the MC group but it did not reach significance. There were not significant differences with respect to sex ratio, location, presence of adenomas, T-intraepithelial lymphocytes (TIL), Crohn-like peritumoral reaction, mean age, tumor size, and percentage of mucinous or cribriform patterns.

Molecular Findings

Table 2 summarizes molecular findings. The percentage of Ki-67 proliferative index was significantly lower in the MC group (p=0.007). The difference in the expression of P53 and MMR genes by immunohistochemistry did not reach significance.

A significantly higher proportion of P53 mutations was detected in the MC group (p=0.039). However, no difference in the presence of KRAS or BRAF mutations was observed between carcinomas with and without micropapillary pattern. The MIN status of these two groups of carcinomas was similar using either the NCI microsatellite panel (p=0.614) or the panel with 11 microsatellites (p=0.686).

DISCUSION

MC has been previously described as an aggressive variant of carcinoma with a high incidence of lymphovascular invasion, nodal and distant metastasis and poor clinical outcome in various locations; nevertheless the study of this variant in colorectal carcinomas has been limited to less than 100 cases and its molecular profile of has yet to be fully defined. We present a series of 337 colorectal carcinomas including 27 cases with at least 10% micropapillary component. In agreement with the two series previously published, we also find a significantly greater proportion of cases with lymph node and distant metastasis as well as a higher number of affected lymph nodes. Furthermore, we have also found a highly significant relation between the presence of MC and the extent of invasion with all our cases being either T3 or T4. The grade and overall stage were also significantly higher in the MC group.

Vascular (thin-walled and venous vessel) and perineural invasion were significantly more frequent in carcinomas with MC component.

MSI-H in colorectal carcinomas has been associated with good prognosis and typically relates with expansive growth pattern, high Ki-67 proliferative index and absence of P53 mutation. All our 27 MC carcinomas presented an infiltrative growth pattern and a significantly higher proportion of P53 mutations and a lower Ki-67 proliferative index. Nevertheless, no significant association between the presence of MC component and the absence of MSI-H was found in our series, neither by the study of the expression of the MMR genes or by direct assessment with two microsatellite panels.

An issue still to be solved is the importance of the proportion of MC component needed to establish a MC phenotype in colorectal carcinoma. In this series we have considered our cut-off at 10%, however 30 cases (included in the non-MC group) presented a MC component inferior to 10% of the tumor volume and are being subject of further studies.

CONCLUSIONS

Colorectal MC appears to be more aggressive than conventional colorectal adenocarcinoma. MC present at a higher tumor stage with frequent lymphovascular and perineural invasion and nodal metastasis. They also have more tendency to carry P53 mutations.

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Table 1: Univariate analysis to correlate clinicopathological features with MC.

CATEGORICAL VARIABLE	% MC >=10% (N= 27)	% MC <10% (N = 310)	P-VALUE
Gender			0.420
Male	18 (66.6)	179 (57.7)	
Female	9 (33.3)	131 (42.3)	
Location			0.683
Proximal	11(40.7)	111 (35.8)	
Distal	16 (59.3)	190 (61.3)	
Unknown	0	9 (2.9)	
Configuration			0.068
Ulcerated / Stenosing	20 (74.1)	169 (54.5)	
Polipoid / Exophytic	7 (25.9)	138 (44.5)	
Unknown	0	3 (1.0)	
Histology			0.552
Adenocarcinoma	25 (92.6)	269 (86.8)	
Mucinous adenocarcinoma	2 (7.4)	41 (13.2)	
Grade (WHO)			**0.017**
1	7	157	
2	15	93	
3	5	60	
Extent of invasion (pT)			**<0.001**
pT1	0	17 (5.5)	
pT2	0	42 (13.5)	
pT3	7 (25.9)	154 (49.7)	
pT4	20 (74.1)	97 (31.3)	
Stage			**<0.001**
1	0	51 (16.5)	
2	2 (7.4)	117 (37.7)	
3	19 (70.4)	113 (36.5)	
4	6 (22.2)	29 (9.3)	
Lymph node involvement (pN)			**<0.001**
0	2 (7.4)	174 (56.1)	
1	10 (37.0)	73 (23.5)	
2	15 (55.6)	63 (20.3)	
Growth pattern			**<0.001**
Infiltrative	27	205 (66.1)	
Expansive	0	103 (33.2)	
Unknown	0	2 (0.7)	
Cytologic grading			**0.027**
High-grade	19 (70.4)	146 (47.1)	
Low or intermediate grade	8 (29.6)	163 (52.6)	
Unknown	0	1 (0.3)	
Peritoneal invasion			**<0.001**
Present	18 (66.7)	92 (29.7)	
Absent	9 (33.3)	218 (70.3)	
Metastasis (pM)			**0.047**
Present	6 (22.2)	29 (9.4)	
Absent	21 (77.8)	281 (90.6)	
Thin-walled vessel invasion			**<0.001**
Present	27	134 (43.2)	
Absent	0	175 (56.5)	
Unknown	0	1 (0.3)	
Venous vessel invasion			**<0.001**
Present	16 (59.3)	70 (22.6)	
Absent	11 (40.7)	240 (77.4)	
Perineural invasion			**<0.001**
Present	12 (44.4)	40 (12.9)	
Absent	15 (55.6)	270 (87.1)	
Crohn-like lymphoid reaction			0.134
Present	5 (18.5)	107 (34.5)	
Absent	22 (81.5)	203 (65.5)	
TIL			0.226
Present	1 (3.7)	42 (13.6)	
Absent	26 (96.3)	267 (86.1)	
Unknown	0	1 (0.3)	
Adenomas			0.101
Present	10 (37.0)	72 (23.2)	
Absent	16 (59.3)	233 (75.2)	
Unknown	1 (3.7)	5 (1.6)	
NUMERICAL VARIABLE	**% MC >=10 (N= 27) MEAN ± SD**	**% MC <10 (N = 310) MEAN ± SD**	**P-VALUE**
Age (years)	65.8 ± 11.8	68.7 ± 12.0	0.176
Tumor size (mm maximum)	36.7 ± 10.7	43.4 ± 20.9	0.126
Solid carcinoma (%)	16.7 ± 18.0	8.6 ± 18.1	<0.001
Mucinous carcinoma (%)	6.9 ± 15.6	13.2 ± 25.8	0.584
Cribriform structures (%)	7.0 ± 13.0	8.3 ± 16.9	0.911
Nodal involvement (n)	4.5 ± 3.3	2.1 ± 4.0	<0.001

Table 2: Univariate analysis to correlate molecular features with MC.

MOLECULAR VARIABLE	% MC >=10 (N= 27)	% MC <10 (N = 310)	P-VALUE
Ki-67 proliferative index (%) *	47.6 ± 19.2	59.0 ± 21.2	**0.007**
p53 accumulation			0.227
Present	18 (66.7)	163 (52.6)	
Absent	9 (33.3)	146 (47.1)	
Unknown	0	1 (0.3)	
P53 mutation			**0.039**
Present	22 (81.5)	188 (60.6)	
Absent	5 (18.5)	118 (38.1)	
Unknown	0	4 (1.3)	
P53 altered (mut. and/or accum.)			0.134
Present	22 (81.5)	204 (65.8)	
Absent	5 (18.5)	104 (33.6)	
Unknown	0	2 (0.6)	
17p loss			0.389
Present	11 (40.7)	137 (44.1)	
Absent	13 (48.1)	104 (33.6)	
Unknown	3 (11.1)	69 (22.3)	
18q loss			1
Present	18 (66.7)	181 (58.4)	
Absent	8 (29.6)	88 (28.4)	
Unknown	1 (3.7)	41 (13.2)	
MSI (NCI panel)			0.614
MSS	19 (70.4)	240 (77.4)	
MSI-L	2 (7.4)	20 (6.5)	
MSI-H	1 (3.7)	33 (10.6)	
Unknown	5 (18.5)	17 (5.5)	
MSI (NCI + 17p and 18q panel)			0.686
MSS	19 (70.4)	217 (70.0)	
MSI-L	2 (7.4)	43 (13.9)	
MSI-H	1 (3.7)	28 (9.0)	
Unknown	5 (18.5)	22 (7.1)	
MMR loss of expression			1
Present	1 (3.7)	21 (6.8)	
Absent	26 (96.3)	279 (90.0)	
Unknown	0	10 (3.2)	
KRAS mutation			1
Present	11 (40.7)	130 (41.9)	
Absent	16 (59.3)	175 (56.5)	
Unknown	0	5 (1.6)	
BRAF V600E mutation			0.167
Present	4 (14.8)	29 (9.4)	
Absent	22 (81.5)	275 (88.7)	
Unknown	1 (3.7)	6 (1.9)	