UNIVERSITÀ DEGLI STUDI DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Medicina (DIMED)

CORSO DI DOTTORATO DI RICERCA IN: ONCOLOGIA CLINICA E SPERIMENTALE E IMMUNOLOGIA

CICLO XXXI°

TITOLO TESI

The value of histology, tumor infiltrating lymphocytes, and mismatch repair status as risk factors of nodal metastasis in screening detected and endoscopically removed pT1 colorectal cancers.

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ABSTRACT

BACKGROUND

The number of patients with colorectal cancers (CRCs) invading the submucosa (pT1) resected during colonoscopy is increasing due to the screening. Such tumors are potentially metastatic, but only 15% of patients have nodal involvement. Histologic criteria currently used for selecting patients needing resection are imprecise and most patients are overtreated. Tumor infiltrating lymphocytes (TILs) and mismatch repair (MMR) status impact on CRC prognosis and could be risk factors of nodal metastasis.

AIM

To identify patients requiring completion surgery, the value of histologic variables, TILs, and MMR status as risk factors of nodal metastasis was investigated in screening detected and endoscopically removed pT1 CRCs.

MATERIALS AND METHODS

Histologic variables, CD3+ and CD8+ TILs, and MMR status were assessed in 102 endoscopically removed pT1 CRC. Univariate and multivariate analyses were used to evaluate the correlation with nodal metastasis.

RESULTS

Positive resection margin, evidence of vascular invasion and tumor budding, wide area of submucosal invasion, and high number of CD3+ TILs were associated with nodal metastasis in

univariate analyses. Vascular invasion was statistically independent in multivariate analysis. Evidence of neoplastic cells in the vessels and/or at the excision border featured 5 out of 5 metastatic tumors and 13 out of 97 non-metastatic ones.

CONCUSIONS

Completion surgery should be mandatory only for patients with pT1 CRC with vascular invasion or with tumor cells reaching the margin. In all other cases, the treatment choice should be entrusted to the evaluation of the risk-benefit ratio of each patient considering the rarity of nodal metastasis.

BACKGROUND

Colorectal cancer (CRC) is among the most common cancer for incidence and mortality in high income countries, where, however, screening programs are proving effective in reducing mortality rates.(1) Indeed, CRC cases and deaths can be prevented through endoscopic removal of precancerous lesions and early cancers in asymptomatic patients with positive fecal occult blood test (FOBT).(2) Consequently, the number of CRC infiltrating the submucosa (TNM staged as pT1) detected and removed during colonoscopy is increasing.(3) The management of such tumors is somehow still undefined because they are potentially metastatic, thus completion surgery would be necessary to assess nodal status, but the frequency of nodal metastasis is low (about 15% of cases).(4) Moreover, up to 20% of patients may experience post-operative complications, thus the risk-benefit ratio may be unfavorable.(5) To reduce the number of unneeded bowel resections, operation is indicated only in patients with high risk of nodal metastasis according to the features of their tumors.(6) However, the histologic variables used vary among countries and in Europe seem to be little stringent.(6, 7) The establishment of reliable criteria for the identification of patients needing surgery is crucial.

In addition to resection margin, vascular invasion, and tumor differentiation, several other histologic features have been proposed so far.(6, 8) The most promising seems to be tumor budding (a morphologic surrogate of epithelial-mesenchymal transition, a mechanism through which neoplastic cells acquire motility and invasiveness) and those measuring tumor microscopic extension (*i.e.* depth, width, and area of the submucosal invasion).(4, 8-13)

Number and type of tumor infiltrating lymphocytes (TILs) in CRC have been reported to influence tumor behavior and patients' prognosis.(14) Higher levels of CD3+ and CD8+ TILs have been found in CRCs without vascular invasion compared to cases with vessel infiltration.(15)

Moreover, quantification of CD3+ and CD8+ lymphocytes has been demonstrated to be a valid predictor of tumor recurrence and survival.(16-18)

CRC with mismatch repair (MMR) deficiency have been associated with a better prognosis and with a longer disease free and overall survival compared to MMR-proficient CRC.(19-21) Moreover, a lower risk of nodal and distant metastasis at diagnosis have been reported in CRC with impaired MMR system, independently of the pathologic features of the tumor.(22) It has also been observed that poor differentiation worsens the prognosis of CRC patients only if associated with MMR proficiency, suggesting that the MMR status of the tumor should be included in the grading of CRC to better stratify patients.(23)

Since both a low amount of CD3+ and CD8+ TILs and MMR proficiency have been associated with a worse CRC prognosis in terms of survival, the hypothesis is that these variables could be correlated also to nodal metastasis.

AIM

The aim of this study was to investigate the value of histologic variables, CD3+ and CD8+ TILs, and MMR status as risk factors of nodal metastasis to aid in the clinical decision-making process whether to operate or not patients with screening detected and endoscopically removed pT1 CRC.

MATERIALS AND METHODS

Patients

This retrospective study was conducted on 102 consecutive asymptomatic and FOBT positive patients with a pT1 CRC endoscopically resected as a whole (*i.e.* fragmented cases were excluded) during the period 2009-2015 among the CRC screening program of Padua. According with European guidelines, patients had been divided into low risk of nodal metastasis (48 patients) and high risk of nodal metastasis (64 patients).(6) Completion surgery was performed in 63 patients (52 patients of the high risk group and 11 patients of the low risk group who opted for the operation). Seven high risk patients refused the bowel resection and five were not eligible for surgery due to comorbidities. Follow-up schedules for all the patients are summarized in Table 1.(24-26) None of the patients had distant metastasis at the time of the initial diagnosis. All procedures were performed accordingly with the 1964 Helsinki declaration and its later amendments. This study adheres to REMARK guidelines.(27)

Table 1. Follow-up schedules of the groups of patients.

Variable	Non-operated patients	Operated patients (non-metastatic)	Operated patients (metastatic)
Clinical visit	After 3 mos and then every yr for 5 yrs	Every 6 mos for 5 yrs	Every 3 mos for 2 yrs and then every 6 mos for 3 yrs
Colonoscopy	After 3 mos and then every yr for 5 yrs	After 1, 2, and 5 yrs	After 1, 2, 5, and 10 yrs
Serum CEA and CA 19-9	After 3 mos and then every yr for 5 yrs	Every 6 mos for 5 yrs	Every 3 mos for 2 yrs and then every 6 mos for 3 yrs
Thoracoabdominal contrast-enhanced CT	After 3 mos and then every yr for 5 yrs	Every yr for 5 yrs	Every 6 mos for 3 yrs and then every yr for 2 yrs
Abdominal US	After 2 and 4 yrs	-	-

CEA = carcinoembryonic antigen; CT = computed tomography; US = ultrasonography.

Histology

Information regarding tumor location (divided into right, transverse, and left colon, and sigmoid colon-rectum), type (pedunculated and non-pedunculated tumor), and size (expressed in cm) were recovered from the histological reports. The H&E-stained original slides of each case were reviewed to evaluate the variables described below. Resection was regarded as incomplete when invasive tumor cells were observed at or within 1 mm of the excision margin (identified by ink or diathermy effect).(6) This data was further refined by recording also if the cells reached the resection border. Cases in which incorrect sample orientation did not allow a sure recognition of the margin were recorded as not evaluable. Unlike WHO classification, histologic grade was determined by the less differentiated area of the invasive cancer regardless of its amount and it was divided into low and high grades.(28, 29) Vascular invasion was defined as presence of tumor cells within endothelial-lined channels. Tumor budding, a single tumor cell or a cell cluster consisting of four tumor cells or less at the invasive front of the cancer, was assessed according to Lugli and colleagues using Leica DM4000 B microscope (Leica Biosystems, Newcastle upon Tyne, UK) with an objective magnification of 20x and an eyepiece FN diameter of 22 (normalization factor used = 1.210) and recorded both as present/absent, number of buds, and budding category (Bd 1 = 0-4 buds; Bd 2 = 5-9 buds; Bd 3= 10 or more buds).(30) The depth of submucosal invasion was evaluated according to the criteria reported by Kawachi and colleagues and expressed in mm.(9) In pedunculated lesions with infiltration of the submucosa confined to the head of the polyp, the depth was considered 0 mm. The width and the area of submucosal invasion were assessed following the definition of "maximum width of carcinoma" and "total area of submucosal invasion by carcinoma" used by Toh and colleagues and were expressed in mm and mm^2, respectively.(10) These last three variables were evaluated on virtual slides using the Aperio ImageScope viewing software (Leica Biosystems) on virtual slides obtained by scanning the H&E-stained original slides with a Leica SCN400 Slide Scanner (Leica Biosystems).

Immunohistochemistry

Immunohistochemistry was performed as described elsewhere on 4 μm thick consecutive sections from the most representative FFPE sample of each case with the primary antibodies listed in Table 2.(31) MLH1, MSH2, MSH6, and PMS2 immunoreactions were interpreted following the criteria stated by Shia and colleagues to assess MMR system status.(32, 33)

Table 2. Antibodies used in the study.

Antibody	Clone	Company	Dilution
CD3	LN10	Leica Biosystems, Newcastle upon Tyne, UK	1:100
CD8	C8/144B	Agilent Technologies, Santa Clara, USA	1:50
MLH1	ES05	Agilent Technologies, Santa Clara, USA	1:25
MSH2	FE11	Agilent Technologies, Santa Clara, USA	1:25
MSH6	EP49	Agilent Technologies, Santa Clara, USA	1:25
PMS2	EP51	Agilent Technologies, Santa Clara, USA	1:25

Image analysis

All the CD3 and CD8 immunostained slides were scanned with a Leica SCN400 Slide Scanner (Leica Biosystems). Virtual slides were imported in the Visiopharm image analysis environment (version 4.5.6.5; Visiopharm, Horsholm, Denmark). The consecutive CD3 and CD8 virtual slides of each case were aligned using the Tissuealign add-on (Visiopharm), thus providing precise stacking of the tissue sections. The region of interest (ROI), *i.e.* the area of submucosal invasion of the tumor, was outlined on each CD3 immunostained virtual slide and automatically reproduced by the program on the aligned CD8 immunostained virtual slide. Automated quantification of CD3+ and CD8+ lymphocytes within the ROI, expressed as number and density (number of

immunostained cells per mm^2), was achieved using a custom-made algorithm created with the Visiopharm software.

Statistical analysis

Fisher's exact test was used to investigate associations between categorical variables and groups of patients. Normality of the distribution of the continuous variables was assessed by both graphical (boxplot and qq-plot) and formal methods (Shapiro-Wilk test). Without normality, Wilcoxon rank-sum test with continuity correction was used to assess significant differences between groups of patients. Receiver-operating characteristic (ROC) curve and the area under the ROC curve (AUC) were used to assess the ability of selected variables to differentiate between groups of patients. Youden procedure was applied to determine the best threshold value. All the variables that were significantly correlated with nodal metastasis in the univariate analyses were considered as covariates in a multivariate penalized logistic regression model. Model performance was assessed with ROC curve and AUC. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the best combination of risk factors were calculated. A $p<0.05$ was considered statistically significant. All statistical analyses were performed using the R software (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Clinical findings

Patients were 61 males and 41 females, with mean age of 62.2±6.1 years. The mean length of the 63 resected bowel tracts was 18.1±10.6 cm and the mean number of harvested lymph nodes was 7.5±5.6. In 9 cases (resection length range: 5.5-13.0 cm) less than 12 lymph nodes were found, although the perivisceral fat was completely included; in 4 of these cases, no lymph nodes were detected. Residual disease was found in 3 resections, nodal metastasis in 4 cases, and in one case both residual tumor and nodal metastasis were present.

The mean follow-up duration was 48.7±23.0 months in the non-operated group and 50.1±22.5 months in the operated one. Two patients left the follow-up after 2 and 4 months of surgery (no residual tumor and nodal metastasis were present in their resections). One patient of the non-operated group had a local tumor relapse detected by colonoscopy after 4 years. No patients had nodal or distant metastasis or died during the follow-up period.

Univariate analyses of nodal metastasis

Results of the univariate analyses of nodal metastasis (present in 5 out of 102 patients) are summarized in Table 3. Incomplete endoscopic resection was correlated with nodal metastasis ($p<0.05$). Tumor cells reached the excision margin in 3 out of 5 metastatic tumors, were observed at a distance less than 1 mm in one of these cases, and in the last one the resection was complete. The rate of tumors with vascular invasion (Figure 1) was greater in the group with nodal involvement than in that without it (80.0% and 7.2%, respectively; $p<0.05$). Tumor budding (Figure 1), regardless of the recording mode (*i.e.* presence of buds, number of buds, or tumor budding category), was associated with nodal metastasis (all $p<0.05$). As for tumor budding

category, Bd1 was compared with Bd2 and Bd3 grouped together due to the paucity of cases in these last classes. Depth of submucosal invasion was higher in tumors with nodal metastasis than in those without it, however the difference was not statistically significant. The minimum depth of submucosal invasion in metastatic tumors was 3.4 mm; 45 non-metastatic tumors had higher values. Eighty-nine tumors without nodal involvement were deeper than the threshold of 1 mm.(7) ROC curve was plotted using the depth of submucosal invasion measurements and the calculated AUC was 0.74. The best threshold value obtained was 4.9 mm; 3 tumors with nodal metastasis and 26 tumors without nodal metastasis were deeper than this value. An increased area of submucosal invasion was correlated with nodal metastasis ($p<0.05$). The minimum area of submucosal invasion in metastatic tumors was 55.2 mm^2; 15 non-metastatic tumors had higher values.

The number of CD3+ TILs (Figure 2) was higher in tumors with nodal involvement than in those without it ($p<0.05$). The AUC of CD3+ TILs was 0.87 and the best threshold was 95,045 lymphocytes; all the metastatic and 26 non-metastatic tumors had higher values. Despite density of CD3+ TILs was higher in the metastatic tumors than in the non-metastatic ones, the difference was not statistically significant. Sex, age, tumor location, type, and size, histologic grade, width of submucosal invasion, MMR status, and number and density of CD8+ TILs (Figure 2) were not associated with nodal metastasis. All the tumors with nodal metastasis were low grade and MMR-proficient, thus it was not possible to investigate the prognostic value of the histologic grade integrated with the MMR status.

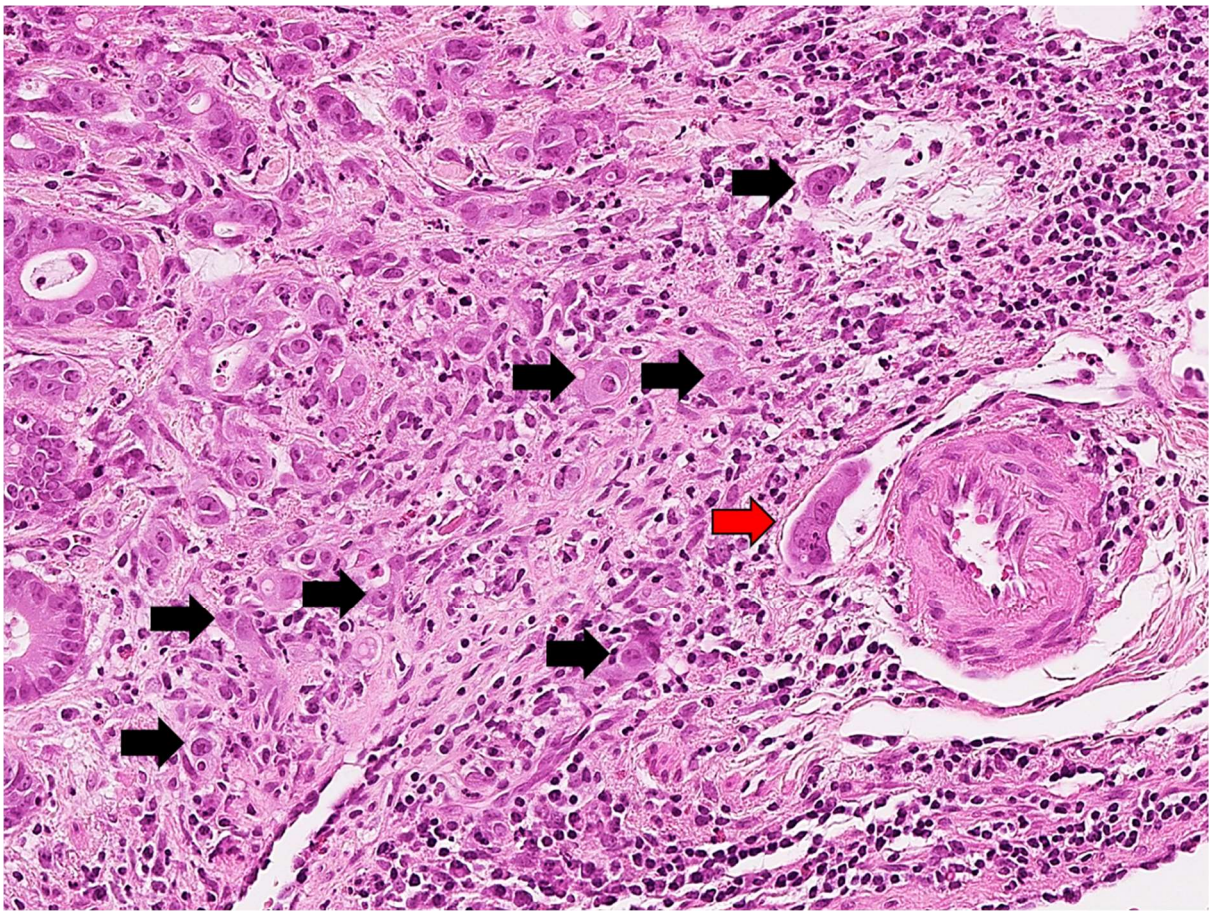

Figure 1. Photomicrograph of one of the endoscopically removed colorectal cancer infiltrating the submucosa included in this study showing tumor buds (*black arrows*) and vascular invasion (*red arrow*). Original magnification 400x.

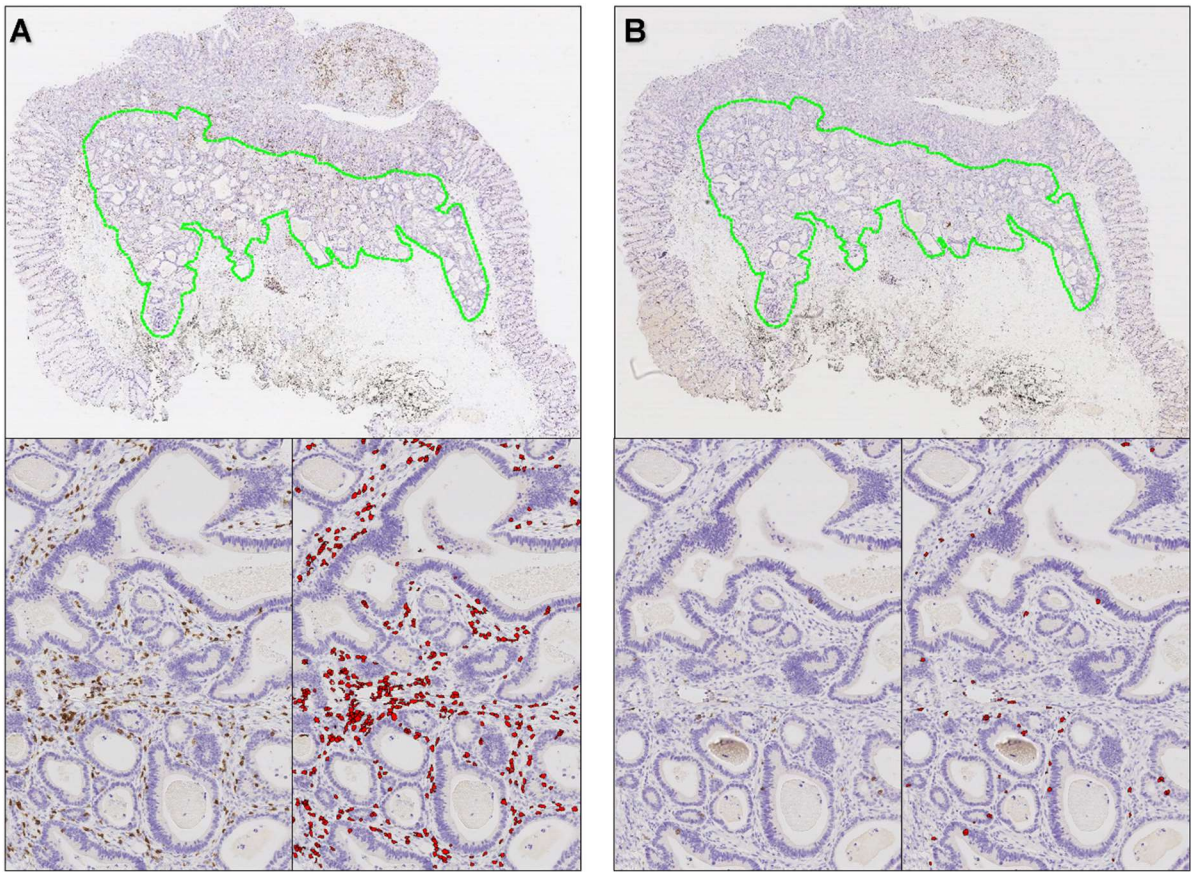

Figure 2. The images show the region of interests (*green line*) outlined in the CD3 (**A**) and CD8 (**B**) immunostained virtual slides and the automated quantification of the lymphocytes (*in red in the insets*) of one of the endoscopically removed colorectal cancer infiltrating the submucosa included in this study. Original magnification 20x and 400x.

Table 3. Univariate analysis of all the variables for nodal metastasis.

Variable	Negative for nodal metastasis (%)	Positive for nodal metastasis (%)	*p*-value
Number of patients	97 (95.1)	5 (4.9)	
Sex			
Male	59 (96.7)	2 (3.3)	
Female	38 (92.7)	3 (7.3)	NS
Age (years)			
Mean±s.d.	62.2±6.1	60.8±6.8	NS
Tumor location			
RC/TC/LC/SR	6/3/5/83	0/0/0/5	NS
Tumor type			
Pedunculated	40 (95.2)	2 (4.8)	
Non-pedunculated	58 (95.0)	3 (5.0)	NS
Tumor size (cm)			
Mean±s.d.	1.8±1.0	1.6±0.3	NS
Resection margin			
Not evaluable	3 (100)	0 (0.0)	
>1 mm	55 (98.2)	1 (1.8)	
≤1 mm	39 (90.7)	4 (9.3)	<0.05
Histologic grade			
Low grade	84 (94.7)	5 (5.3)	
High grade	13 (100)	0 (0.0)	NS
Vascular invasion			
Absent	90 (99.0)	1 (1.0)	
Present	7 (63.6)	4 (36.6)	<0.05
Tumor budding			
Absent	81 (97.6)	2 (2.4)	
Present	16 (84.2)	3 (15.8)	<0.05
Number of buds			
Mean±s.d.	1.0±3.0	2.6±2.5	<0.05
Tumor budding category			
Bd1	92 (96.7)	3 (3.3)	
Bd2	2 (50.0)	2 (50.0)	
Bd3	3 (100.0)	0 (0.0)	<0.05[a]
Depth of submucosal invasion (mm)			
Mean±s.d.	4.0±2.4	5.3±1.9	NS
Width of submucosal invasion (mm)			
Mean±s.d.	6.3±3.6	7.1±2.3	NS
Area of submucosal invasion (mm^2)			
Mean±s.d.	39.5±36.6	73.0±32.7	<0.05
Mismatch repair (MMR) status			
MMR-proficiency	89 (94.7)	5 (5.3)	
MMR-deficiency	8 (100.0)	0 (0.0)	NS

CD3+ TILs number			
Mean±s.d.	83,417.9±128,468.7	168,657.5±61,750.1	<0.05
CD3+ TILs density (number/mm²)			
Mean±s.d.	1,942.9±1,585.3	2,839.9±2,128.2	NS
CD8+ TILs number			
Mean±s.d.	14,755.1±27,201.3	27,855.3±22,504.2	NS
CD8+ TILs density (number/mm²)			
Mean±s.d.	331.2±260.5	375.8±245.3	NS

RC = Right colon; TC = Transverse colon; LC =Left colon; SR = Sigmoid colon-rectum; NS = Not significant; TIL = Tumor infiltrating lymphocyte.

[a] Bd1 compared with Bd2 and Bd3 grouped together.

Multivariate analysis of nodal metastasis

Multivariate analysis by a penalized logistic regression model using resection margin, vascular invasion, tumor budding category, area of submucosal invasion, and number of CD3+ TILs showed that nodal metastasis was independently correlated only with presence of vascular invasion (Table 4). Vascular invasion was evident in 4 out of 5 metastatic CRCs and in 7 out of 97 non-metastatic ones. Adding the status of resection margin, defined as evidence of tumor cells at the resection border, guarantees the identification of all the metastatic cases (5 out of 5) minimizing the number of those without metastasis (13 out of 102). This combination of risk factors showed a sensitivity of 100%, a specificity of 87%, a PPV of 28%, and a NPV of 100%.

Table 4. Multivariate analysis of the five significative variables for nodal metastasis using a penalized logistic regression model.

Variable	Estimate	95% Confidence Interval	*p*-value
Vascular invasion	3.07	-0.37-11.03	<0.05
Resection margin	-1.63	-6.41-0.66	NS
Tumor budding category	2.23	-1.77-11.66	NS
Area of submucosal invasion	0.02	-0.19-0.12	NS
CD3+ TILs number	0.01	0.01-0.02	NS

NS = Not significant

Correlations between variables

Presence of buds, high number of buds, and high tumor budding categories were strongly associated with vascular invasion (all $p<0.05$). Number and density of CD3+ TILs were correlated neither with vascular invasion nor with MMR status. Contrary to vascular invasion, MMR-deficiency was significantly correlated both with high number and density of CD8+ TILs ($p<0.05$). Histologic grade was not associated with MMR status.

DISCUSSION AND CONCUSIONS

Nodal metastasis in pT1 CRC is infrequent, accounting for about 15% of cases.(4) In this series its prevalence was 4.9%, in line with previous findings indicating that in non-Asian populations it can be slightly lower.(4) Using the current European criteria for the risk assessment of nodal metastasis, more than half of the patients underwent completion surgery.(6) However, only 5 out of the 63 operated patients had nodal metastasis, thus in 92.0% of cases there was an overtreatment strongly highlighting the need to adopt more reliable criteria. Moreover, in 9 resections less than 12 lymph nodes (the minimum number for applying TNM staging) were harvested, thus the intervention was useless from this point of view.(34) These resections were much shorter than the others. Anyway, neither nodal nor distant metastases were found during the follow-up.

As for residual disease, in all the 3 cases without nodal involvement the CRCs had been endoscopically removed from the sigmoid colon-rectal region; these tumors were low grade and lacked vascular invasion, however their distance from the excision border was equal to or less than 1 mm. Probably in these cases a mucosectomy would be more appropriate than a surgical resection. In the remaining case, the sigmoid-rectal tumor was well differentiated, with a free resection margin, but with neoplastic cells in the vessels, thus the probability of nodal metastasis was high and surgery the right option. The only event found in this CRC screening population during the follow-up was a local tumor recurrence after 4 years. The previous pT1 tumor was low grade, without vascular invasion, but with a resection margin not evaluable. The patient refused both the intervention and the mucosectomy (the more prudent choices) by opting for the follow-up.

Concerning histologic variables, our results confirmed the association between nodal metastasis and positive resection margin, evidence of vascular invasion, presence of tumor budding, and wide area of submucosal invasion.(4, 8-12, 35-37) This makes sense because tumor

cells reach the lymph nodes through lymphatic vessels, thus vascular invasion is a prerequisite for nodal metastasis and its detection is a cornerstone in defining the metastatic risk of a tumor. However, cases occur in which vascular invasion is not observed in histology, despite the presence of metastasis in the draining lymph nodes. Indeed, histology evaluates one or more internal plane of a three-dimensional lesion, but it may happen that the visualized sections did not sampled (and therefore documented) the zone with vascular invasion. Similarly, when a pT1 CRC is incompletely removed during colonoscopy, a part of the lesion cannot be analyzed histologically. This part of the tumor could have already infiltrated the vessels or, if left in place, could metastasize in the future. In the metastatic process, tumor budding is the upstream phase of vascular invasion and, in this series, there was a strong correlation between tumor budding and vascular invasion. Through the mechanism of epithelial-mesenchymal transition, cancer cells are thought to acquire motility and invasiveness, to detach from the tumor mass, to infiltrate the extracellular matrix as single cells or small clusters (the tumor buds), and to penetrate in the lymphatic vessels.(4, 12) According to the literature, our findings showed that a single tumor bud already confers an increased risk of nodal metastasis and that this is greater the more the tumor buds are numerous.(4, 8, 9, 11, 12) Tumor budding categorization is a practical and standardized method to quantify this variable and is reliable in identifying cases with a higher risk of nodal involvement.(4, 30) In this series of pT1 CRCs, there was a significant correlation between the area of submucosal invasion and nodal metastasis. Tumors with larger area of infiltration are more likely to encounter the lymphatic vessels of the upper third of the submucosa (where the density of the vessels is higher) and thus have more chances to invade them.(10, 38) Our results showed that 15 CRCs without nodal involvement had an area larger than the smallest one among the metastatic cases. Moreover, the median area of submucosal invasion of tumors with nodal metastasis reported by Toh and colleagues was smaller than the median in this series (54.1 mm^2 and 65.0 mm^2, respectively).(10) Despite the available data are too few to draw conclusions, it seems likely that the number of false positive and false negative cases setting a threshold for this variable will be too high to be useful

in clinical practice . Moreover, a precise measurement of the area is time consuming, since it requires image analysis of virtual slides. Another limit is that depth, width, and area of submucosal invasion are all affected by the sampling of the gross specimen. Indeed, the point and the angle of the cut can have an enormous impact on these measurements.(39) Contrary to literature, in this study neither depth nor width of submucosal invasion were correlated to nodal metastasis, despite the measurements were higher in the metastatic group.(8-10) A possible explanation could be the higher rate of pedunculated tumor in our series; long stalks can host deep and narrow tumor infiltrations. Anyway, about 90% and 50% of our CRCs, respectively, exceeded the commonly used depth (1 mm) and width (5 mm) thresholds for assessing the tumor metastatic risk. Moreover, these methods are quite subjective (*e.g.* positioning the "Haggitt" lane) and difficult to apply (*e.g.* mucosal ulceration).(8, 9) Contrary to literature, histologic grade was not associated with nodal metastasis, despite the less stringent criteria for assigning a high grade.(8, 28, 29) The fact that all the metastatic CRCs were low grade demonstrates that differentiation of the tumor not necessarily corresponds to its disseminating ability. Anyway, this result impaired part of this study because it was not possible to test the prognostic value of the combination between high histologic grade and MMR proficiency. Despite all the 8 MMR-deficient CRCs were in the non-metastatic group of patients, MMR status was not correlated with nodal involvement, contrary to the findings of Malesci and colleagues.(22) CRCs with MMR deficiency carried higher number and density of CD8+ TILs compared to MMR-proficient cases, as found by other authors.(40, 41) In MMR-deficient tumors, known to have a high mutagenic potential, the accumulation of mutations leads to the production of neo-antigens, enhancing the anti-tumor immune response.(41-43) Surprisingly, a higher number of CD3+ TILs was found in CRCs with nodal metastasis compared to those without it. This is the opposite of what has been reported in literature so far, where it has been shown that an intense immune response was usually associated with a better prognosis in terms of survival.(14-18) Moreover, Pages and colleagues have found that increased levels of CD3+ TILs were correlated with absence of vascular invasion, a data not confirmed in this

series.(15) Anyway, the quantification of CD3+ TILs seems inappropriate to select patients for completion surgery since about 30% of cases (including all the metastatic ones) had values higher than the best threshold. The number of CD3+ TILs is increased in such tumors, but they may be ineffective. Indeed, metastatic tumors could have implemented immune escape mechanisms producing immunosuppressive molecules.(44) Otherwise, these lymphocytes could be mostly T helper 17 (Th17) cells, a subset of pro-inflammatory T-lymphocytes characterized by the production of interleukin 17 that has been correlated with nodal metastasis and poor prognosis in CRC.(45, 46)

Evidence of vascular invasion was the only statistically independent factor by multivariate analysis, but it failed in identifying a metastatic tumor. Thus, the addition of another variable was required. A selection system based on the detection of tumor cells in the vessels or at the excision margin would have prevented 45 unnecessary resections without losing any metastatic tumor. Thus, completion surgery should be mandatory only in cases with these features. In all other cases, given the rarity of nodal metastasis, mucosectomy and follow-up or follow-up alone can be valid alternative to completion surgery, and a treatment decision should be taken by the multi-disciplinary team after an appraisal of the risk-benefit ratio of each patient.

A strength of this study lies in the homogeneous features of the investigated population: i) patients were consecutive, demographically similar, and all asymptomatic with positive FOBT at the time of inclusion; ii) initial treatment was the same for all; iii) scheduled follow-up was strictly observed; iv) abandonment rate was 1.9% and only after surgery. Variables were analyzed only in non-surgical specimens (analogy of material), adhering to their definitions and evaluating methods (comparability of results), and, when possible, using image analysis technology (objectivity of result). The main weaknesses concern the retrospective setting and the limited number of collected cases that did not allow to perform some evaluations. Indeed, additional larger studies are required

to confirm these findings, to better characterize TILs in relation to nodal involvement and to explore if MMR-proficient high grade CRCs have an increased risk of nodal metastasis.

In conclusion, positive resection margin, presence of vascular invasion, high tumor budding categories, large area of submucosal invasion, and high number of CD3+ TILs are risk factors of nodal metastasis in screening detected and endoscopically removed pT1 CRCs. In the clinical decision-making if completion surgery is required or not, it seems that resection is mandatory only for tumor in which neoplastic cells reach the excision border or are found inside the vessels. In all other cases, multi-disciplinary team should choose the treatment after the evaluation of the risk-benefit ratio of each patient considering that nodal metastasis is rare.

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