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ANATOMICAL PATHOLOGY

High reproducibility is attainable in assessing histoprognostic parameters of pT1 colorectal cancer using routine histopathology slides and immunohistochemistry analyses

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Summary

Assessment of the risk of lymph node invasion and tumour recurrence is critical to determine whether additional surgery is required in patients with endoscopically-removed pT1 colorectal cancer (CRC). A reproducible assessment of this risk of recurrence based on histopathological parameters is crucial for relevant therapeutic decisions. The inter-observer reproducibility of these parameters was the subject of our study. Two pathologists independently analysed 163 endoscopically-removed pT1 CRC recorded in a local digestive cancer registry database (Finistère, France). Using haematoxylin-eosin-saffron (HES) and immunohistochemistry slides, they evaluated several parameters related to the risk of tumour recurrence according to the international pT1 CRC-dedicated guidelines.

Based on Kappa and intra-class correlation coefficients, good to very good inter-observer agreement was obtained by analysing vertical and lateral margins, submucosal invasion, tumour differentiation and lymphovascular invasion. The reproducibility of tumour budding quantification was only fair on the basis of HES slides but reached a very good agreement using cytokeratin immunohistochemistry. Dual colour cytokeratin and podoplanin immunohistochemistry also improved inter-observer agreement for the detection of lymphovascular invasion. All patients with loco-regional nodal metastases (7 of 101 who underwent complementary surgery) or distant metastases (3 patients) were diagnosed as having a high risk of recurrence and requiring an additional surgery by the two observers. Our study showed that good to very good inter-observer agreement is achievable in evaluating the pathological parameters of recurrence risk in endoscopically-removed pT1 CRC. In addition to HES slides, the detection of lymphovascular invasion and tumour budding can benefit with more reproducible immunohistochemical analyses.

Key words: Early colorectal cancer; lymphovascular invasion; tumour budding; immunohistochemistry; reproducibility.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in males and the second in females, with an estimated 1.4 million cases and 693,900 deaths occurring in 2012. Screening for CRC has substantially contributed to the downward trends in CRC incidence and mortality over the last 2 decades.[1] Most CRC arise from a non-malignant lesion, via a multistep process involving a series of histological, morphological, and genetic changes that accumulate over time, in a process that takes 7–15 years. Early detection and removal of precancerous polyps are critical to interrupt the progression from adenoma to carcinoma and, thus, to prevent the development and spread of CRC. The change of colorectal cancer incidence and mortality rates is attributed to the increase in colorectal cancer screening and adenoma removal.[2,3] With the widespread use of endoscopic resection techniques and the introduction of cancer screening programs, the detection of malignant polyps has increased.[4,5]

A malignant colorectal polyp is a lesion in which neoplastic cells have invaded through the muscularis mucosae into the submucosa. Early CRC is defined as invasion into the submucosa but not into the muscularis propria. They are classified as pT1 tumours in the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) tumour node metastasis system,[6,7] and account for 0.75–5.6% of colorectal polyps removed in general diagnostic colonoscopy practice.[8] Lymph node invasion is found in 10–15% of patients with early CRC.[4,5] In

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this way, surgical resection with regional lymphadenectomy has long remained the standard treatment for early CRC.[9] Nevertheless, since at least 85% of patients with early CRC do not have regional nodal involvement, it is important to avoid surgical treatment and its associated not negligible morbidity and mortality in patients at low risk of recurrence, and to perform complementary surgery only in patients at high risk of further CRC progression. The distinction between low risk and high risk early CRC with its consequences in terms of therapeutic decision has been the field of several studies and consensus guidelines over the past 15 years with emphasis on several prognostic parameters. These histopathological prognostic parameters, evoked for example in consensus guidelines such as the Paris classification and the recommendations of the Japanese Society for Colorectal Cancer (JSCCR), are now listed in the standardised pathological report of early CRC diagnosed in polypectomy pieces proposed by the French Society of Pathology. They include the status of the resection margin, differentiation grade, Haggitt (for pedunculated polyps) or Kudo–Kikuchi (for sessile polyps) classification, invasion depth and width using the method proposed by Ueno *et al.*, lymphovascular invasion and tumour budding.[5,10–21]

Indeed, the diagnosis and management of early CRC require the expertise of endoscopists for resections and the expertise of gastrointestinal pathologists for histopathological diagnosis. Beyond the diagnosis of cancer, pathologists are in charge of evaluating the above-mentioned histopathological prognostic markers that must appear in the pathological report to distinguish between low risk and high risk early CRC and to permit further clinico-pathological correlation and management. After an endoscopic resection of a polyp, the indication of an additional colectomy must balance the risk of lymph node involvement and local recurrence against the risk of morbidity and mortality following surgical excision. The decision-making process must be discussed at a multidisciplinary team meeting on the basis of these histopathological criteria. The informed choice of the patient must also be taken into consideration.

An accurate and standardised evaluation of the different histopathological prognostic parameters is required for homogeneous and relevant clinico-pathological correlation. Nevertheless, even using standardised methodologies, inter-observer reproducibility to evaluate the different prognostic parameters remains uncertain and can cause dilemmas in the therapeutic decision. Several reproducibility studies, most of the time focusing on a single prognostic parameter (e.g., tumour budding or lymphovascular invasion) and more rarely analysing several parameters together, have reported substantial inter-observer variations with only poor to moderate inter-observer reproducibility for the different prognostic parameters listed above.[22–32] This lack of reproducibility could considerably modify the therapeutic decision from one histopathological interpretation to another.

This study was performed as part of our laboratory quality program in the field of digestive pathology. The objective was to analyse the interobserver reproducibility in the evaluation of histo-prognostic parameters of endoscopically-removed pT1 malignant polyps. The potential impact on the therapeutic decision was also discussed retrospectively.

MATERIAL AND METHODS

Case selection

The cases included in this study were all the locally-removed pT1 CRC (i.e., endoscopy and trans-anal resection) diagnosed from 1 January 2009 to 31 December 2013 in the area of Finistère (France, population 899,870 in 2011). All cases were recorded in the digestive cancer registry database that gathers information on patient's demographics, tumour characteristics, clinical and pathological staging, treatment modalities, recurrence and survival. Information was obtained from pathologists, general practitioners, hospitals, and private physicians (gastroenterologists, surgeons, and oncologists). The quality and exhaustiveness of the registry are certified every 4 years by an audit of the French National Committee of Registries. Formalin fixed, paraffin embedded (FFPE) tumour samples of pT1 CRC were collected from pathology laboratories' archives on the basis of initial pathology reports and slides review. The present study complied with our national and institutional guidelines. All samples were included in a registered tumour tissue collection and the study was conducted in accordance with the Declaration of Helsinki following approval by our institutional review board (CHRU Brest, CPP no. DC – 2008–214).

Sample processing and immunohistochemistry

Tissue sections of 4 μm were used to produce new haematoxylin-eosin-saffron (HES) and immunohistochemistry (IHC) slides. IHC analyses were performed using the Ventana Benchmark XT automated slide preparation system (Ventana-Roche Diagnostics, France) using the ultraView Universal DAB Detection Kit (Ventana-Roche Diagnostics) and the ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana-Roche Diagnostics). For each tumour sample, we produced one cytokeratin IHC slide (DAB revelation, clone AE1/AE3, 1:50 dilution; Dako, Denmark), one dual colour IHC slide with cytokeratin (Red revelation) and podoplanin (DAB revelation, clone D2-40, pre-diluted; Dako) and one dual colour IHC slide with cytokeratin (Red revelation) and CD31 (DAB revelation, clone IC/70A, 1:20 dilution; Dako).

For dual colour IHC, the slides underwent an antibody denaturation step at 95°C for 8 min, after incubation with the first antibody (requiring DAB revelation, i.e., podoplanin or CD31 antibodies in our study) and before the incubation with the second antibody (requiring Red revelation, i.e., cytokeratin antibody in our study). The cytokeratin IHC slides underwent a single incubation cycle and DAB revelation.

Methodology of evaluation of individual parameters

Each slide was read independently by two pathologists (FB and LD), without prior knowledge of the pathological report or clinical context. The two observers and practitioners of surgical pathology in the pathology department of the University Hospital of Brest were trained in digestive pathology and were used to the analysis of early CRC slides according to French national guidelines.[15] The following parameters were evaluated only for the invasive adenocarcinoma component (i.e., not adjacent intraepithelial neoplasia or carcinoma *in situ* when present). Micrometric calibrated eyepieces were used for the measurements.

Beside the total thickness of the tumour, the method by Ueno *et al.* was used for the measurement of submucosal invasion depth and width.[19] The depth of the tumour submucosal invasion was measured from the superficial aspect to the deepest part of the invasion when the muscularis mucosae could not be identified. When the muscularis mucosae could be identified, it was used as the upper yardstick of the submucosal layer (see Fig. 1 for examples) The width was a measurement of the invasive front.[4,19] For pedunculated polyps, Haggitt's classification was also used scoring the depth of invasion from level 1 to level 4: level 1 in cases with invasive carcinoma limited to the head of the polyp, level 2 in cases with carcinoma invading the neck of the polyp, level 3 in cases with carcinoma invading the stalk of the polyp, and level 4 in cases with carcinoma invading into the submucosa below the stalk of the polyp.[16] For sessile tumours, a staging system adapted from the three level Kudo and Kikuchi system (SM1, SM2, SM3) through the classification of Paris (i.e., SM1 corresponds to submucosal invasion <1000 μm) was used to classify the depth of submucosal invasion in SM1 (<1000 μm), SM2

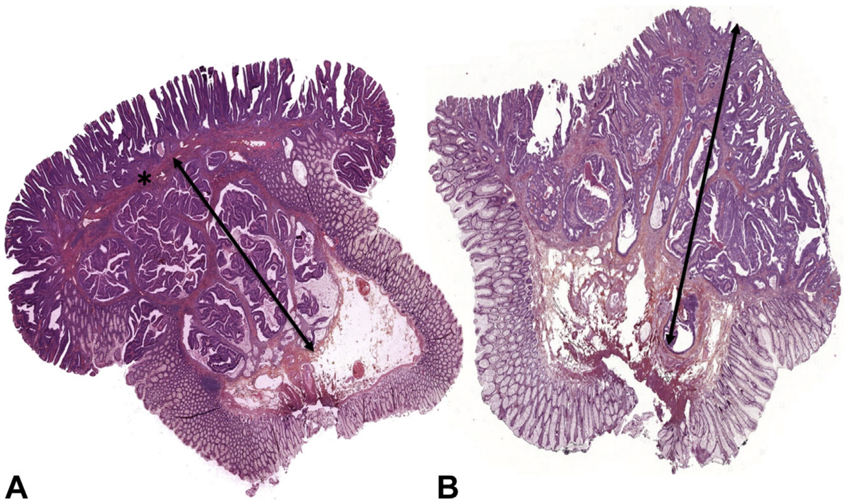

Fig. 1 Examples of measurement of invasion depths (double-headed arrows) on HES slides. (A) The muscularis mucosae (*) was present and used as the upper yardstick of the submucosal layer to measure the deepest submucosal invasion. (B) When the muscularis mucosae could not be identified, invasion was measured from the superficial aspect to the deepest part of the invasion.

(1000–2000 μm) or SM3 (>2000 μm) levels.[10,17,18] Each pathologist decided which cases were suitable for Haggitt's or SM1–SM3 classifications.

Each pathologist also decided whether the measurement of the vertical and lateral margins could be assessed or not. For the resection margin status, when assessable, R0 and R1 statuses were concluded if the tumour was located >1 mm (R0) or ≤1 mm (R1) from the resection margin, respectively.[33,34] The distance between the tumour and the margin was measured in millimeters vertically and laterally. The presence of intraepithelial neoplasia or of *in situ* carcinoma on the lateral margins was also noted.

For the grade of differentiation, we used the four grade classification given by the World Health Organization: grade 1 for well-differentiated adenocarcinoma with more than 95% gland formation, grade 2 for moderately differentiated adenocarcinoma with 50–95% gland formation, grade 3 for poorly differentiated adenocarcinoma with less than 50% gland formation and grade 4 for undifferentiated carcinoma lacking any gland formation or mucin production. Mucinous adenocarcinoma and signet-ring cell adenocarcinoma were individualised when the percentage of the corresponding tumour component was greater than 50%.[35] The lymphovascular invasion was first concluded as present or absent on the basis of HES sections and, in another independent analysis, on dual colour IHC sections coupling epithelial marker (cytokeratin) and vascular markers (podoplanin and CD31). Lymphovascular invasion images were also counted per tissue section using HES and IHC slides. Tumour perineural infiltration was also noted as present or absent on the basis of HES sections.

Finally, tumour budding was quantified following the recommendations for reporting tumour budding of the International Tumour Budding Consensus Conference 2016.[20] Tumour budding was defined as isolated single cells or clusters of cancer cells (<5 cells without gland formation) at the front of the tumour. Pathologists first searched for the area with the highest tumour budding at low magnification and then counted the number of buds within this 'hot spot' area to finally express their density in terms of buds per 0.785 mm^2 surface. Tumour budding was scored as follows: grade 1, low grade, in cases with 0–4 buds per 0.785 mm^2 surface; grade 2, medium grade, in cases with 5–9 buds per 0.785 mm^2 surface; and grade 3, high grade, in cases with ≥10 buds per 0.785 mm^2 surface. HES sections were first used in evaluating for tumour budding before a second evaluation was performed using cytokeratin IHC slides.

Impact of inter-observer agreement/disagreement on therapeutic decision

For management of patients after endoscopic resection of a polyp with pT1 adenocarcinoma, a decision whether or not to perform additional surgery is made after weighing the risk of morbidity and mortality of the surgery versus the risk of potential local recurrence, lymph node invasion and distant metastasis on the basis of pathological parameters. Although the risks of patient morbidity and mortality that weighted the decision were not retrospectively assessable in our study, we attempted to evaluate retrospectively the potential impact of the inter-observer agreement/disagreement on the therapeutic decision in patients with endoscopically-removed pT1 CRC. In this way, we took into account the pathological criteria proposed by the Japanese Society for the Cancer of the Colon and the Rectum (JSCCR) and the European Society for Gastrointestinal Endoscopy (ESGE) who advocate complementary surgery if at least one of the five parameters listed below is noted: (1) a positive vertical margin (R1), (2) submucosal invasion depth >1000 μm, (3) grade 3–4 tumour differentiation including signet-ring cell carcinomas and mucinous carcinomas, (4) grade 2–3 tumour budding, (5) the presence of lymphovascular invasion.[11,14]

We retrospectively combined these criteria into a 'combined risk assessment' (CRA) score from 0 (i.e., no criterion present, signifying a low risk tumour and surgery not indicated) to 5 (five criteria present, surgical treatment being indicated in patients with scores 1–5 with high risk tumours) to estimate potential variations in the therapeutic decision between the results of the two pathologists. Given that only patients with no positive criteria could safely avoid complementary surgery with almost no risk of cancer recurrence, when one of the criteria was not evaluable on the tissue sections, we chose to consider the hypothesis with the poorest prognosis and to count 1 point for this criterion in our study.

Statistical analyses

Statistical analyses were performed using MedCalc Statistical Software version 13.2.2 (MedCalc Software, Belgium). The level of significance was set at $p < 0.05$. The weighted Kappa statistic test (quadratic weights) was used to quantify inter-observer agreement for R0/R1 status of the vertical and lateral margins, level of submucosal invasion according to Haggitt's and SM1–SM3 classifications, tumour differentiation, presence of tumour perineural invasion, presence of lymphovascular invasion on HES slides and IHC slides, tumour budding grading on HES slides and IHC slides, and CRA scores using only HES slides and using HES combined with IHC slides. Intraclass correlations coefficients (for single measures reflecting the reliability of the ratings for one, typical, single observer, and for average measures reflecting the reliability of the ratings for the two observers averaged together) and Bland–Altman plots graphs were used to calculate the inter-observer agreements for the measurements of vertical and lateral margins (mm), of total tumour thickness, of submucosal invasion depth and width (μm) and the number of lymphovascular invasion images per HES and IHC sections. The values of Kappa strength agreements and intra-class correlations were interpreted as follows: <0.20 poor, 0.21–0.40 fair, 0.41–0.6 moderate, 0.61–0.80 good and 0.81–1.00 very good agreement.[36]

RESULTS

Cases included

We identified 312 patients with pT1 CRC diagnosed between 1 January 1 2009 and 31 December 2013 in the area of Finistère, France, and listed in the database of the digestive cancer registry. For 19 patients, tumour material was too limited to perform additional tissue sections for IHC analyses and these cases were excluded. We also excluded 130 patients with pT1 CRC diagnosed on colorectal surgical specimens. In this way, we finally included 163 locally-removed pT1 CRC tumour samples in our study, from 104 (63.8%) males and 59 (36.2%) females with a mean age of 67 years (range 43–93 years). Among these pT1 CRC, nine (5.5%) cases occurred in the right colon, 13 (7.8%) in the left colon and 140 (85.9%) in the recto-sigmoid (no location for 1 case). Twenty samples (12.3%) were obtained by trans-anal resection and 143 (87.7%) by endoscopy. The size of the polyps ranged from 5 to 90 mm (mean size 19 mm). The polyps were pedunculated in 81 (49.7%) cases and sessile in 82 (50.3%) cases. An *en bloc* resection was achieved in 143 (87.7%) cases, whereas a piecemeal resection was performed in 20 (12.3%) cases.

At the time of our study in 2018, nine (5.5%) patients had a local recurrence of the tumour. A total of 101 (62%) patients underwent subsequent colorectal surgery with lymphadenectomy and seven (4.3%) of them had regional nodal metastases. Distant metastases were observed in three (1.8%) patients, with a CRC-related death in one case (0.6%).

Inter-observer reproducibility of individual parameters

The details of Kappa and intra-class correlations coefficients are listed in Tables 1 and 2 and Bland–Altman plot graphs

Table 1 Summary of results and agreement between the two observers: qualitative and semi-quantitative parameters

Qualitative/semi-quantitative parameters	Results	Observer 1	Observer 2	Kappa [95% confidence intervals] quality of agreement and no. of differences
Vertical margin R0/R1 status	R0	95 (58.3%)	95 (58.3%)	Kappa=0.854 [0.757–0.951]
	R1	63 (38.7%)	62 (38%)	Very good
	NA	5 (3.1%)	6 (3.7%)	12 (7.3%) differences
Vertical margin tissue	Healthy tissue	134 (82.2%)	139 (85.3%)	Kappa=0.864 [0.739–0.989]
	Invasive carcinoma	24 (14.7%)	18 (11%)	Very good
	NA	5 (3.1%)	6 (3.7%)	7 (4.3%) differences
Lateral margin R0/R1 status	R0	90 (55.2%)	112 (68.7%)	Kappa=0.869 [0.809–0.930]
	R1	31 (19%)	9 (5.5%)	Very good
	NA	42 (25.8%)	42 (25.8%)	26 (15.9%) differences
Lateral margin mucosa	No neoplasia	114 (69.9%)	116 (71.2%)	Kappa=0.833 [0.698–0.968]
	Invasive carcinoma	7 (4.3%)	5 (3.1%)	Very good
	LG IEN	9 (5.5%)	13 (8%)	10 (6.1%) differences
	HG IEN	7 (4.3%)	1 (0.6%)	
	NA	26 (16%)	28 (17.2%)	
Haggitt's classification level of submucosal invasion	Level 1	27 (16.6%)	29 (17.8%)	Kappa=0.976 [0.962–0.990]
	Level 2	21 (12.9%)	22 (13.5%)	Very good
	Level 3	29 (17.8%)	26 (16%)	11 (6.7%) differences
	Level 4	0	0	
	NA	86 (52.8%)	86 (52.8%)	
SM1–SM3 level of submucosal invasion	SM1	27 (16.6%)	18 (11%)	Kappa=0.918 [0.872–0.964]
	SM2	23 (14.1%)	25 (15.3%)	Very good
	SM3	23 (14.1%)	28 (17.2%)	25 (15.3%) differences
	NA	90 (55.2%)	92 (56.4%)	
Tumour differentiation	Grade 1	45 (27.6%)	39 (23.9%)	Kappa=0.791 [0.682–0.899]
	Grade 2	92 (56.4%)	103 (63.2%)	Good
	Grade 3 NOS	7 (4.3%)	8 (4.9%)	25 (15.3%) differences
	Signet-ring cell ca	1 (0.6%)	1 (0.6%)	
	Mucinous ca	18 (11%)	12 (7.4%)	
Perineural invasion	Absence	160 (98.2%)	160 (98.2%)	Kappa=0.66 [0.219–1]
	Presence	3 (1.8%)	3 (1.8%)	Moderate
				2 (1.2%) differences
Lymphovascular invasion per section (HES)	Absence	151 (92.6%)	150 (92%)	Kappa=0.61 [0.377–0.843]
	Presence	12 (7.4%)	13 (8%)	Good
				9 (5.5%) differences
Lymphovascular invasion per section (cytokeratin and CD31 IHC)	Absence	156 (95.7%)	155 (95.1%)	Kappa=0.79 [0.56–1]
	Presence	7 (4.3%)	8 (4.9%)	Good
				3 (1.8%) differences
Lymphovascular invasion per section (cytokeratin and podoplanin IHC)	Absence	131 (80.4%)	126 (77.3%)	Kappa=0.908 [0.829–0.987]
	Presence	32 (19.6%)	37 (22.7%)	Very good
				5 (3.1%) differences
Tumour budding grading (HES sections)	Grade 1 (0–4 buds)	159 (97.5%)	145 (89%)	Kappa=0.235 [−0.0561–0.527]
	Grade 2 (5–9 buds)	3 (1.8%)	11 (6.7%)	Fair
	Grade 3 (≥10 buds)	1 (0.6%)	7 (4.3%)	19 (11.7%) differences
Tumour budding grading (cytokeratin IHC sections)	Grade 1 (0–4 buds)	117 (71.8.5%)	93 (57.1%)	Kappa=0.842 [0.778–0.906]
	Grade 2 (5–9 buds)	17 (10.4%)	36 (22.1%)	Very good
	Grade 3 (≥10 buds)	29 (17.8%)	34 (20.9%)	30 (18.4%) differences

Ca, carcinoma; HES, haematoxylin-eosin-saffron; HG IEN, high grade intra-epithelial neoplasia; IHC, immunohistochemistry; LG IEN, low grade intra-epithelial neoplasia; NA, not applicable; NOS, not otherwise specified; R0, >1 mm margin; R1, ≤1 mm margin; SM1–SM3, three tiers submucosa invasion levels.

Table 2 Summary of results and quality of agreement between the two observers: quantitative parameters

Quantitative parameters	Intraclass coefficients [95% confidence intervals] and quality of agreement	
Vertical margin (mm)	Single measures	0.9501 [0.9321–0.9635] Very good
	Average measures	0.9744 [0.9649–0.9814] Very good
Lateral margin (mm)	Single measures	0.5786 [0.4455–0.6867] Moderate
	Average measures	0.7331 [0.6164–0.8143] Good
Total tumour thickness (mm)	Single measures	0.8506 [0.8006–0.8888] Very good
	Average measures	0.9192 [0.8892–0.9411] Very good
Submucosal invasion depth (μm)	Single measures	0.7949 [0.7230–0.8497] Good
	Average measures	0.8857 [0.8392–0.9188] Very good
Submucosal invasion width (μm)	Single measures	0.9117 [0.8806–0.9349] Very good
	Average measures	0.9538 [0.9365–0.9664] Very good
Lymphovascular invasion per section (HES)	Single measures	0.3757 [0.2360–0.5002] Fair
	Average measures	0.5462 [0.3819–0.6669] Moderate
Lymphovascular invasion per section (cytokeratin and CD31 IHC)	Single measures	0.8071 [0.7461–0.8567] Good
	Average measures	0.8933 [0.8546–0.9216] Very good
Lymphovascular invasion per section (cytokeratin and podoplanin IHC)	Single measures	0.8732 [0.8311–0.9053] Very good
	Average measures	0.9323 [0.9078–0.9503] Very good

HES, haematoxylin-eosin-saffron; IHC: immunohistochemistry.

are illustrated in Fig. 2 for quantitative data. For the vertical and lateral margins, inter-observer reproducibility was very good for determination of R0/R1 status as well as for histopathological characterisation of the tissue at vertical and lateral margins (i.e., healthy tissue, low grade or high grade intra-epithelial neoplasia or invasive carcinoma). Reproducibility was moderate (lateral margin) to very good (vertical margin) for measurements of the margins.

Inter-observer agreement was also very good for classification of depth of invasion using Haggitt's and SM1–SM3 systems, and it was good to very good for the measurement of submucosal invasion depth, width and total tumour thickness.

It should be noted that beyond this good to very good reproducibility, determination of vertical and lateral margin statuses as well as level-based classification and measurements of vertical invasion were judged to be not feasible in about half of the samples by the two observers (e.g., for piece meal resection specimens or due to problems with sample orientation in pathology laboratories).

Inter-observer agreement was good for tumour differentiation including the diagnosis of signet-cell ring carcinoma and mucinous carcinoma. It was moderate for the diagnosis of tumour perineural invasion.

Inter-observer agreement for evaluation of lymphovascular invasion was moderate to good using HES slides or the dual colour cytokeratin and CD31 IHC slides and it was very good using dual colour cytokeratin and podoplanin IHC slides (see Fig. 3 for examples). The same trend was observed considering on the one hand the binary absence/presence of lymphovascular invasion, and on the other hand the number of lymphovascular invasion images identified per tissue section.

For the semi-quantification of tumour budding, inter-observer agreement was fair using HES slides and very good using cytokeratin IHC slides (see Fig. 3 for examples).

Potential impact of discordant pathological parameters on therapeutic decision

Based on the analyses of each observer, we calculated the aforementioned CRA score for each tumour, using on the one hand only HES slides, and on the other hand HES slides combined with cytokeratin and podoplanin and cytokeratin IHC slides for the diagnosis of lymphovascular invasion and/ or tumour budding, respectively. The inter-observer agreement for CRA-score based therapeutic decision was moderate to good using only HES slides and it was moderate to very good when combining HES slides with IHC slides (see Table 3). The improvement was more marked when using IHC to detect lymphovascular invasion than to detect tumour budding. For both observers, the decision based on the combination of HES slides with IHC slides would have resulted in a slight but not significant increase in the surgical decision rate ($p > 0.05$).

All patients who developed lymph node invasion in their clinical follow-up would have been scored 1 or higher using CRA score by the two observers, as well as the three patients who had distant visceral without nodal metastases, including one patient who did not undergo additional surgery at the time of his real-life clinical management.

DISCUSSION

Early detection and appropriate treatment of a tumour, ideally at non-invasive stage, are crucial in the fight against cancer. This is particularly true in the field of CRC for which the oncogenic sequence from precursor lesions to invasive carcinoma is now well described and has led to screening programs to detect and remove colorectal neoplastic lesions at the earliest possible stage. As a result, the diagnosis of malignant polyps has increased and raised the issue of therapeutic management of patients with endoscopically-removed invasive pT1 CRC. Several prognostic markers, mainly based on histopathology, are now used to predict the risk of metastatic spreading of pT1 CRC. When this metastatic risk is judged to be zero, no additional surgery is indicated, which is not without risks for the patient. Conversely, the higher the metastatic risk, the more the surgical intervention is indicated. The goal of surgery is to minimise the risk of further progression of the cancer, which is more complicated to treat at an advanced stage with a risk of death from cancer. Given the crucial role of the histopathological prognostic parameters in the therapeutic decision, it is important to ensure that the inter-observer reproducibility in the diagnosis of these

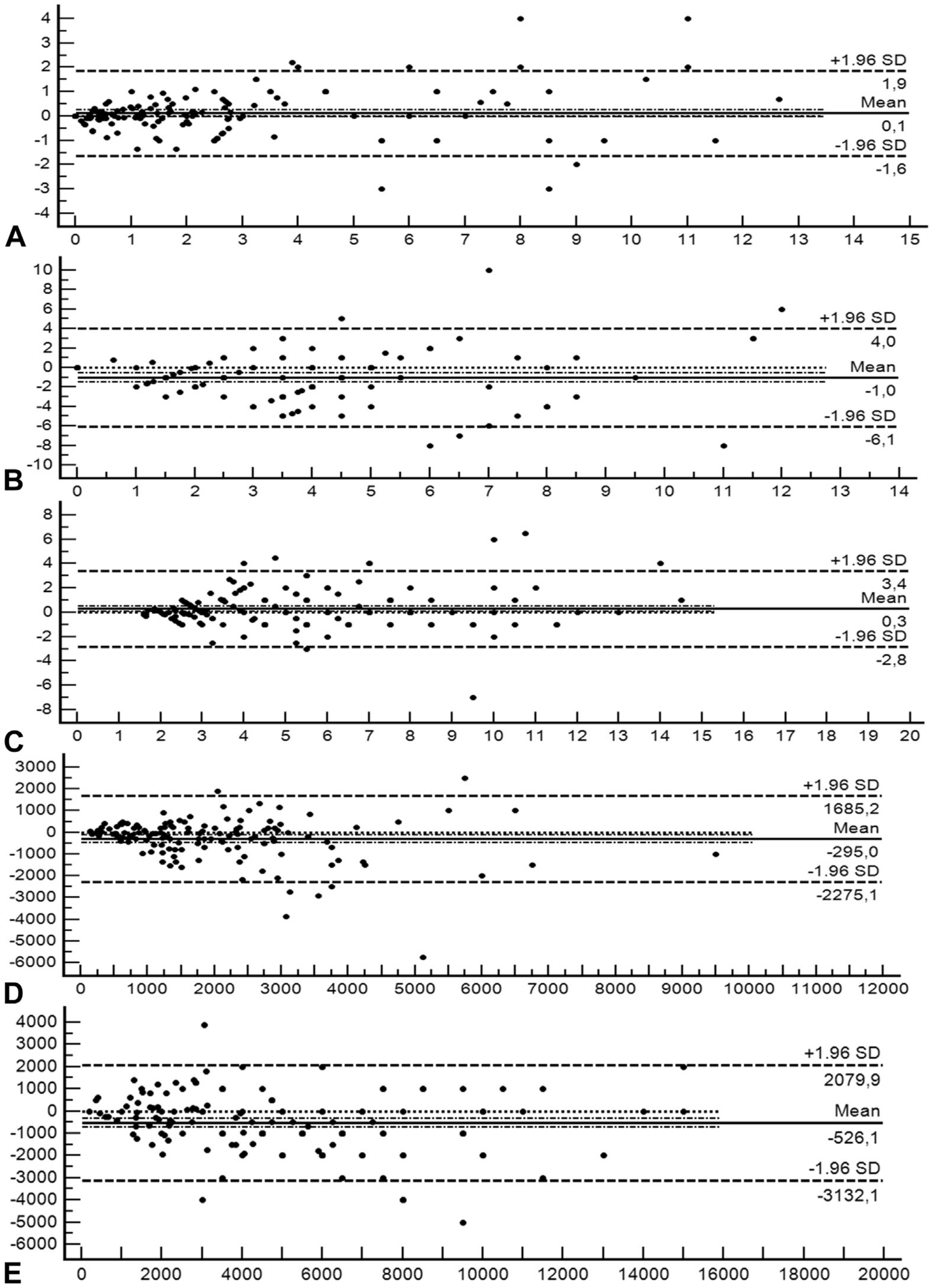

Fig. 2 Bland–Altman plots reflecting the difference between the two measures (vertical axes) in function of the mean of the two measures (horizontal axes) for the two observers for quantitative parameters; mean differences and 95% confidence intervals are also indicated for each parameter. (A) Vertical margin (mm); (B) lateral margin (mm); (C) total tumour thickness (mm); (D) submucosal invasion depth (μm); (E) submucosal invasion width (μm).

parameters is sufficient to allow their widespread use in clinical practice.

In our study, the inter-observer agreement for the diagnosis of the best-established factors associated with a high risk of recurrence [i.e., positive vertical margin (R1); submucosal invasion depth >1000 μm; grade 3–4 tumour differentiation including signet-ring cell and mucinous carcinomas; grade 2–3 tumour budding; presence of lymphovascular invasion] was very good to good, except for tumour budding for which the agreement was only fair using HES slides (see Tables 1 and 2 for detailed values). Keeping in mind that reproducibility studies are difficult to compare given the various material, parameters and methodologies used, our results nevertheless appear to be generally more satisfactory with higher inter-observer agreements than those reported in previous studies.[22–32] In our study, the fact that both observers were used to interpreting pT1 CRC in the same pathology centre with the same guidelines and frequent double evaluations in daily routine practice could at least partly explain this superior performance compared with multicentre studies performed with pathologists from different laboratories

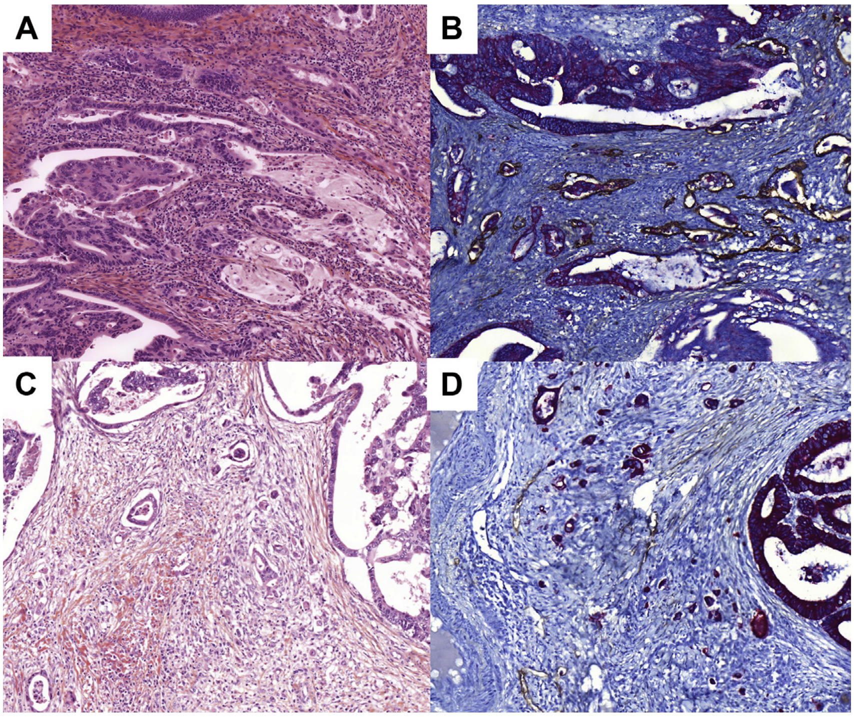

Fig. 3 Examples of (A,B) lymphovascular invasion and (C,D) tumour budding images on (A,C) HES slides and (B,D) dual colour cytokeratin in red (AE1/AE3 clone) and podoplanin in brown (D2-40 clone) immunohistochemistry slides. A and B illustrate the same tumour area as C and D. Lymphovascular invasion and tumour budding are more obvious using immunohistochemistry slides than using HES slides.

Table 3 Summary of results and quality of agreement between the two observers: combined risk assessment score for therapeutic decision faced with real life evolution of patients

Slides used for diagnosis	Combined risk assessment score	Observer 1		Observer 2		Kappa and quality of agreement
		Total	M+	Total	M+	
Using HES sections for tumour budding and lymphovascular invasion	Score 0	28 (17.2%)	–	17 (10.4%)	–	Kappa=0.761 [0.683–0.839] Good
	Score 1	67 (41.1%)	2	70 (42.9%)	1	
	Score 2	47 (28.8%)	5	51 (31.3%)	5	
	Score 3	20 (12.3%)	2	22 (13.5%)	3	
	Score 4	1 (0.6%)	–	3 (1.8%)	–	
	Score 5	0	–	0	–	
	No indication for surgery (score 0)	28 (17.2%)	–	17 (10.4%)	–	Kappa=0.515 [0.328–0.702]
	Indication for surgery (score 1–5)	135 (82.8%)	9	146 (89.6%)	9	Moderate
Using IHC sections for tumour budding (cytokeratin) and HES for lymphovascular invasion	Score 0	21 (12.9%)	–	11 (6.7%)	–	Kappa=0.768 [0.688–0.849] Good
	Score 1	58 (35.6%)	1	47 (28.8%)	–	
	Score 2	48 (29.4%)	5	66 (40.5%)	5	
	Score 3	31 (19%)	3	33 (20.2%)	4	
	Score 4	5 (3.1%)	–	6 (3.7%)	–	
	Score 5	0	–	0	–	
	No indication for surgery (score 0)	21 (12.9%)	–	11 (6.7%)	–	Kappa=0.52 [0.305–0.735]
	Indication for surgery (score 1–5)	142 (87.1%)	9	152 (93.3%)	9	Moderate
Using HES for tumour budding and IHC for lymphovascular invasion (cytokeratin and podoplanin)	Score 0	26 (16%)	–	15 (9.2%)	–	Kappa=0.836 [0.784–0.887] Very good
	Score 1	62 (38%)	2	66 (40.5%)	1	
	Score 2	46 (28.2%)	5	48 (29.4%)	6	
	Score 3	25 (15.3%)	2	25 (15.3%)	2	
	Score 4	4 (2.5%)	–	8 (4.9%)	–	
	Score 5	0	–	1 (0.6%)	–	
	No indication for surgery (score 0)	26 (16%)	–	15 (9.2%)	–	Kappa=0.531 [0.338–0.723]
	Indication for surgery (score 1–5)	137 (84%)	9	148 (90.8%)	9	Moderate
Using IHC sections for lymphovascular invasion (cytokeratin and podoplanin) and tumour budding (cytokeratin)	Score 0	19 (11.7%)	–	11 (6.7%)	–	Kappa=0.834 [0.771–0.896] Very good
	Score 1	58 (35.6%)	1	45 (27.6%)	–	
	Score 2	46 (28.2%)	5	60 (36.8%)	6	
	Score 3	24 (14.7%)	3	29 (17.8%)	3	
	Score 4	15 (9.2%)	–	16 (9.8%)	–	
	Score 5	1 (0.6%)	–	2 (1.2%)	–	
	No indication for surgery (score 0)	19 (11.7%)	–	11 (6.7%)	–	Kappa=0.563 [0.344–0.781]
	Indication for surgery (score 1–5)	144 (88.3%)	9	152 (93.3%)	9	Moderate

HES, haematoxylin-eosin-saffron; IHC, immunohistochemistry; M+, patients with known metastatic evolution in real life (i.e., loco-regional and/or distant).

with different individual habits in the evaluation of pT1 CRC, despite standardised guidelines.

As previously published by some authors, the quantification of tumour budding on HES slides only permitted a fair inter-observer agreement; however, in our study, cytokeratin IHC permitted an increase not only in the detection of tumour budding but also in the inter-observer agreement from 0.235 (fair agreement) to 0.832 (very good agreement) Kappa scores. The value of cytokeratin IHC to determine tumour budding as well as to detect lymphovascular invasion has remained controversial in the literature.[25,27,32] In our opinion, given the results obtained in this study, IHC could also help to improve inter-observer agreement in comparison with HES and it could be of interest for the assessment of lymphovascular invasion (cytokeratin and podoplanin dual colour IHC) and tumour budding (cytokeratin IHC, and although not tested for tumour budding in our study, maybe using cytokeratin and podoplanin dual colour IHC), especially in cases with inter-observer discrepancies based on HES sections. Of note, the improvement in reproducibility was slightly better using cytokeratin and podoplanin (kappa 0.908) in comparison with cytokeratin and CD31 (kappa 0.79); we hypothesise that the specificity of lymphatic vessels and not blood vessels of podoplanin D2-40 clone may have focused the attention of the two pathologists on less numerous vessels in comparison with CD31 IHC, resulting in less laborious and more reproducible detection of emboli. Given the improved inter-observer agreement using IHC slides complementary to HES slides instead of HES slides solely, the prognostic significance of IHC-based parameters in comparison with HES-based parameters could also be a field of interest.

Our reproducibility study has some limitations that will merit further work. As metastases beyond 5 years of follow-up are rare but not impossible, and because the low rates of patients with recurrence and metastases reported in our study prevent us from drawing formal conclusions in terms of prognostic value of the different parameters, it would be interesting in future work to further study the prognostic value of individual and combined as well as HES- and IHC-based parameters. In future studies, it would be also necessary to distinguish between proximal and distal colon (by far the most represented in our study) to determine if the same prognostic parameters would apply throughout the colon.

CONCLUSION

Although there is inevitable inter-observer variability for the appreciation of single factors but also of combined parameters using a CRA score in endoscopically-removed pT1 CRC, the final impact of this variability on the risk of not performing surgery in a patient seems minimal in our series. Of course, and fortunately, many patients who underwent surgery in real life did not develop metastasis.

As the primary goal of decision-making is to avoid metastatic progression in cases of pT1 CRC removed by endoscopy, we think that a more stringent attitude trying to avoid complementary surgery in patients with one (or more) risk factors of cancer recurrence after an endoscopic resection must be considered with caution as the risk of distant loco-regional metastasis, although weak, is real.

Faced with inter-observer variability in the appreciation of prognostic parameters in endoscopically-removed pT1 CRC, interpretation of pT1 CRC HES slides by two pathologists could help to get a consensual result in evaluating the prognostic parameters. Complementary IHC analyses could be also interesting to reach a consensual decision in case of inter-pathologist discrepancy, especially in the field of lymphovascular invasion and tumour budding detection.

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