Human Pathology Reports 31 (2023) 300690

Contents lists available at ScienceDirect

Human Pathology Reports

journal homepage: www.journals.elsevier.com/human-pathology-reports

Original Article

Usefulness of Elastica van Gieson staining and the number of samples prepared for venous invasion of colorectal cancer (pT2–pT4)

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ARTICLE INFO

Keywords:
Colorectal cancer
Vascular invasion
Elastica van Gieson
Whole-tumor sections

ABSTRACT

Aims: Although venous invasion is an important prognostic factor for colorectal cancer, it may be significantly underestimated in actual daily diagnosis. In this study, hematoxylin–eosin (HE)- and Elastica van Gieson (EVG)-stained specimens of colorectal cancer (pT2–pT4) were obtained, and the influence of the number of specimens and the number of EVG-stained specimens on the prognosis were examined.

Methods and results: The presence or absence of venous invasion in 100 colon cancer (pT2–pT4) specimens obtained after surgical resection was observed. Assessments were made by comparing the sections, of either the specimens of the deepest part of the tumor or the whole tumor, stained with only HE, as well as a combination of HE and EVG stains. There was a relative low agreement rate between the assessments made using whole-tumor EVG-stained sections and those employing other methods. With respect to relapse-free survival, no significant difference was observed in the prognosis of cases evaluated using HE-stained samples alone relative to the presence of venous invasion. However, for evaluations made using EVG staining, a significant difference was seen even for deepest-section assessments, and this trend was even stronger when whole-tumor sections were evaluated (EVG [deepest]: $P = 0.0128$, EVG [whole sections]: $P = 0.0069$). When the whole-tumor sections were observed with EVG staining, all 15 cases without venous invasion showed no recurrence within the observation period.

Conclusions: The addition of EVG staining allowed the identification of venous invasion in patients with colorectal cancer, which eventually affects prognosis. Increasing the number of EVG-stained samples improves the possibility of accurate prediction.

1. Introduction

Colorectal cancer is associated with high rates of morbidity and mortality globally [1]. Venous invasion is considered an important prognostic factor along with histological type, wall depth, and presence of organ metastasis [2–6]. The Royal College of Pathologists in the United Kingdom recommends the detection of venous invasions in at least 30 % of cases [7]. However, the utility of venous invasion is considerably underestimated in daily diagnosis [8,9].

Usually, facilities prepare samples with maximal sections, and pathological diagnosis is generally made with hematoxylin-eosin (HE)-stained specimens. Previous studies have reported that the detection rate of venous invasion with Elastica van Gieson (EVG)-stained specimens is higher than that of HE-stained specimens alone [5,9–11]. Although few studies have described EVG staining of all whole-tumor sections, there are reports that this staining protocol of all whole-tumor sections significantly improves the identification of venous invasion [12]. However, clear criteria for the usage and protocols for EVG staining are currently unavailable. Nevertheless, even if only HE staining is used, the detection rate of venous invasion can be increased by

Abbreviations: HE, hematoxylin-eosin; EVG, elastic van Gieson; IMVI, intramural venous invasion; EMVI, extramural venous invasion.

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https://doi.org/10.1016/j.hpr.2022.300690
Received 12 December 2022; Received in revised form 29 December 2022; Accepted 30 December 2022
Available online 5 January 2023

Table 1
Patient demographic information (n = 100).

Factor		
Sex	Male	59
	Female	41
Age (years)	Average (range)	66 (28–87)
Location	Colon	81
	Rectum	19
TNM-Stage	I	19
	II	33
	III	37
	IV	11
T stage	T2	24
	T3	35
	T4	41
Nodal metastasis	negative	56
	positive	44
Tumor diameter	<30 mm	16
	30–60 mm	64
	60 mm<	20
Tumor grade	G1	53
	G2	44
	G3	3
Treatment arm	surgery alone	62
	adjuvant chemotherapy	38
Tumor budding	BD1	76
	BD2	18
	BD3	6

Abbreviations: G, grade; BD, budding;

focusing on the morphological features such as the 'protruding tongue' and 'orphan arteriole [9,12,13]'.

In this study, HE staining and EVG staining of whole-tumor sections of colorectal cancer (pT2–pT4) have been used to examine the influence of the number of specimens and EVG staining on prognosis. In addition, we aimed to verify the optimal handling of the specimens in daily diagnosis.

2. Methods

The study involved 100 consecutive patients aged ≥20 years who underwent surgical resection of primary colorectal cancer between January 1, 2013, and July 31, 2014, at the Department of Surgery, Kurume University Hospital. The study targeted cases of colorectal cancer in whom invasion depth was diagnosed as pT2, pT3, or pT4 by postoperative histological assessments. Cases diagnosed with an invasion depth of pTis and pT1 were excluded because they are expected to have less frequent venous invasion and have a relatively good prognosis. Cases showing postoperative (including endoscopic resection) recurrence or simultaneous or consecutive multiple cancer were excluded. Other exclusion criteria included the following: having the small intestine or appendix as the occupied site; preoperative chemotherapy had been performed, and other cancers recorded within 3 years before surgery (excluding intramucosal cancer).

This study was approved by the Kurume University Life Ethics Committee (Study No. 455).

Table 1 shows the clinicopathological features of 100 patients who underwent surgery.

2.1. Pathological assessment

Tissue blocks were prepared for all sections, and whole-tumor sections were approximately 5 mm in width. Two consecutive sections were cut out from each tissue block and stained with HE and EVG for histological evaluation.

2.2. Pathological diagnosis

Histological evaluations were performed as follows: First, one observer (KN) observed all glass slides of the cases. A second observer (JA) evaluated all of them at the same time as KN while remaining blinded to the clinical information and pathological findings. The presence of venous invasion was determined by careful examination of morphological features using only HE-stained samples. When the venous invasion was suspected, it was confirmed on the EVG-stained specimen, and when the venous invasion was confirmed on the EVG-stained specimen, the presence of HE-stained venous invasion was determined. Cases in which venous invasion could not be recognized in HE-stained specimens and those in which the venous invasion was suspected by HE staining but could not be confirmed by EVG staining were considered to have no HE-stained venous invasion. The results obtained here were defined as the first dataset. Basically, these results were used in following analyses.

EVG-stained specimens were used to determine venous invasion following the criteria developed in previous studies with the consensus of multiple pathologists [14]. Venous invasion was considered to be present when elastic fibers of more than half of the circumference surrounding the tumor nest were stained. This finding confirmed the presence of venous invasion even if an accompanying artery was not found [14]. As for the specimens, the portion of the largest section including the deepest part of the tumor was observed first. Subsequently, each slice of the sections was observed, and the association between the number of section slices to be observed and the detection rate of venous invasion was investigated. The presence or absence of venous invasion was investigated using each of the following observation methods:

(1) Diagnosis using an HE-stained specimen of the portion of the largest section only, which included the deepest part of the tumor (deepest HE).
(2) Diagnosis using the combined EVG- and HE-stained specimens of the portion of the largest section that included the deepest part of the tumor (deepest HE + EVG).
(3) Diagnosis using an HE-stained specimen that included a whole-tumor section (whole-section HE).
(4) Diagnosis using a combined EVG-stained sample and HE-stained whole-tumor section sample (whole-section HE + EVG).

Furthermore, intramural venous invasion (IMVI) was defined as when the deepest part of the venous invasion extended up to the muscularis propria. Cases in which the deepest part was beyond the muscularis propria were considered extramural venous invasion (EMVI). Moreover, we recorded these for each of the four methods. In addition, the morphological characteristics of the venous invasion confirmed in whole-section HE were examined.

2.3. Interobserver study

To examine the interobservers' differences, additional two certificated pathologists observed deepest HE and deepest HE and EVG. The judgment results obtained from two pathologists, SM and MN, were compared with the first data set. By focusing on the morphological features, the judgment by the two pathologists was made by deepest HE, followed by the assessment that was made by deepest HE + EVG. The two newly obtained datasets were compared with the first dataset, which was originally created by KN and JA.

2.4. Analysis methods

We examined the degree of agreement between the diagnostic results in each pair of conditions using Cohen's kappa coefficients. For the 89 patients who did not show distant metastasis at the time of surgery, the relapse-free survival (RFS) rates were calculated using the Kaplan–Meier method. The results were statistically evaluated using a log-rank test. The *P*-values of the RFS rates were Bonferroni-corrected. The venous

Table 2
Comparison of detection rates of venous invasion in four methods.

		EVG(deepest)		
		V(−)	V(+)	
HE(deepest)	V(−)	48	34	82
	V(+)	0	18	18
	Total	48	52	100
kappa coefficient = 0.34 (95 %CI 0.20–0.47)				
		EVG(whole sections)		
		V(−)	V(+)	
HE(deepest)	V(−)	16	66	82
	V(+)	0	18	18
	Total	16	84	100
kappa coefficient = 0.08 (95 %CI 0.03–0.13)				
		EVG(whole sections)		
		V(−)	V(+)	
EVG(deepest)	V(−)	16	32	48
	V(+)	0	52	52
	Total	16	84	100
kappa coefficient = 0.34 (95 %CI 0.20–0.48)				
		EVG(whole sections)		
		V(−)	V(+)	
HE(whole sections)	V(−)	16	35	51
	V(+)	0	49	49
	Total	16	84	100
kappa coefficient = 0.30 (95 %CI 0.18–0.44)				

Abbreviations: HE, haematoxylin–eosin; EVG, Elastica van Gieson; V(−), venous invasion absent; V(+), venous invasion present.

invasion and each clinicopathological factor were analyzed by Cox proportional hazards regression to determine the hazard ratio (HR). The association between the number of EVG stains and the detection rate of venous invasion was estimated by the Kaplan–Meier method. Agreement between pathologists was assessed using the Cohen's κ statistic test. Kappa values between 0.01 and 0.20 were considered slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial, and 0.81–1.00 almost perfect concordance. Statistical analysis was performed using JMP Pro version 15, and in all tests, $P < 0.05$ was considered significantly different.

3. Results

3.1. Comparison of the four methods in detecting venous invasion

The numbers of cases evaluated to have venous invasion by the deepest HE, deepest HE + EVG, whole-section HE, and whole-section HE + EVG methods were 18, 52, 49, and 84, respectively. In terms of the kappa value between whole-section HE + EVG and other observation methods, a minor agreement was observed with the deepest HE, with a value of 0.08. Agreements with deepest HE + EVG and whole-section HE were 0.34 and 0.30, respectively, which indicated only fair agreement (Table 2).

3.2. Relapse-free survival

Observations with HE specimens alone showed no significant difference in the 5-year RFS rate in either the deepest or whole-tumor

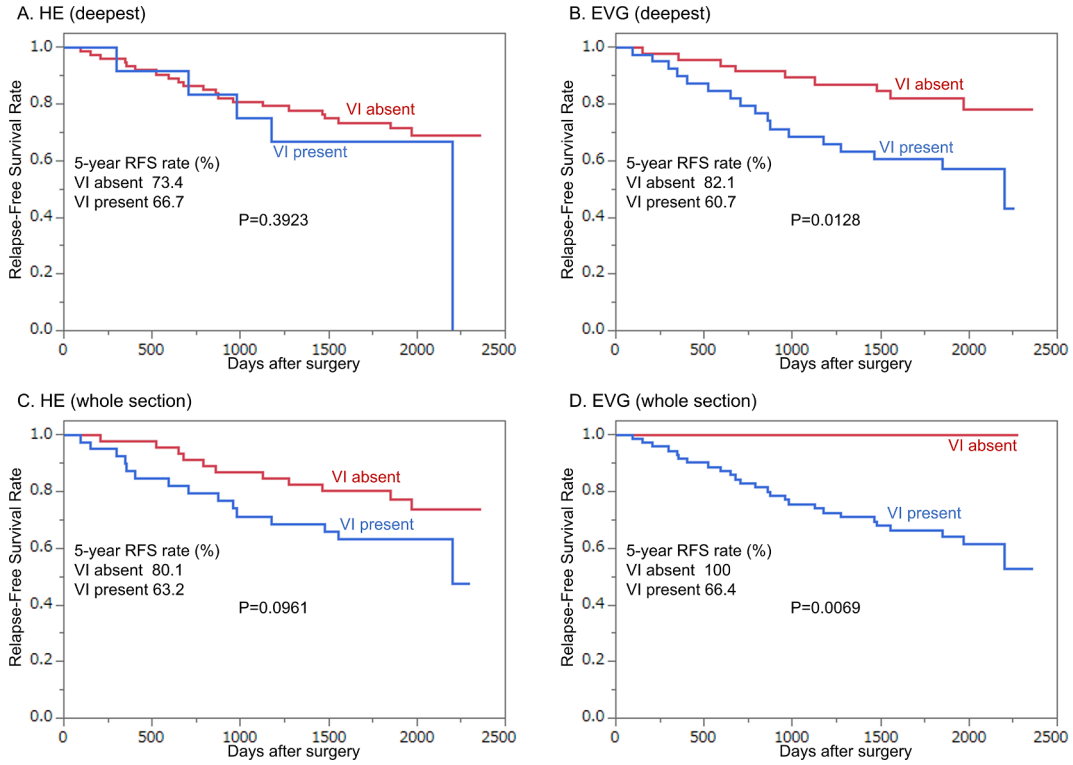

Fig. 1. Association between the presence and absence of venous invasion and the relapse-free survival rate determined by each observation method (89 cases in which distant metastasis was not observed at the time of surgery). A, The association between the presence and absence of venous invasion and the relapse-free survival rate observed in the deepest HE. No significant difference was observed in the prognosis with or without venous invasion ($P = 0.3923$). B, Association between the presence and absence of venous invasion observed in the deepest HE + EVG and the relapse-free survival rate. The prognosis was significantly better in the group without venous invasion than in the group with venous invasion ($P = 0.0128$). C, Association between the presence and absence of venous invasion observed in the whole-section HE and the relapse-free survival rate. No significant difference was observed in the prognosis with or without venous invasion ($P = 0.0961$). D, Association between the presence and absence of venous invasion observed in the whole-section HE + EVG and the relapse-free survival rate. All 15 patients without venous invasion did not show relapse within the observation period, and the prognosis was significantly better than that in the group with venous invasion ($P = 0.0069$). VI, venous invasion.

Table 3
Univariable and Multivariable Analyses of Relapse-Free Survival Using Cox Proportional Hazards Regression Model (For the 89 patients who did not show distant metastasis at the time of surgery).

		Univariable		Multivariable	
Parameter		HR (95 % CI)	P	HR (95 % CI)	P
Tumor grade					
	G1 + G2	Reference		Reference	
	G3	3.64 [0.85–15.59]	0.0818	4.87 [0.94–25.21]	0.0590
T stage					
	T2	Reference		Reference	
	T3	0.81 [0.26–2.53]	0.7193	0.54 [0.16–1.80]	0.3180
	T4	2.01 [0.77–5.25]	0.1530	1.31 [0.45–3.86]	0.6191
Lymphatic invasion					
	Negative	Reference		Reference	
	Positive	1.64 [0.76–3.54]	0.2079	0.84 [0.33–2.17]	0.7210
Adjuvant chemotherapy					
	No	Reference		Reference	
	Yes	1.69 [0.77–3.67]	0.1892	0.80 [0.28–2.33]	0.6889
Venous invasion					
	Negative	Reference		Reference	
	Positive	2.68 [1.20–6.03]	0.0167	2.52 [1.06–5.98]	0.0366
Lymph node metastasis					
	Negative	Reference		Reference	
	Positive	2.04 [0.94–4.41]	0.0710	1.76 [0.59–5.29]	0.3124
Tumor budding					
	BD1 + BD2	Reference		Reference	
	BD3	3.10 [0.92–10.43]	0.0670	1.85 [0.45–7.63]	0.3926

Abbreviations: CI, indicates confidence interval; HR, hazard ratio; G, grade; BD, budding;

section observations (deepest HE: $P = 0.3923$, Fig. 1A; whole-section HE: $P = 0.0961$, Fig. 1C). On the other hand, the 5-year RFS of the group without venous invasion was significantly better in the deepest HE + EVG than that of the group with venous invasion ($P = 0.0128$, Fig. 1B), with a stronger tendency in the whole-section HE + EVG ($P = 0.0069$, Fig. 1D). All 15 patients with an absence of venous invasion in the whole-section HE + EVG did not show relapse during the observation period.

3.3. Univariate and multivariate analyses

In the whole-section HE + EVG, no event occurred in the group without venous invasion; thus, a multivariate analysis thereof was not possible. Instead, the diagnosis result of the deepest HE + EVG, which showed a significant difference in prognosis using the Kaplan–Meier method, was adopted for the presence or absence of venous invasion. The results indicated that the venous invasion-positive group, as assessed by EVG, had a significantly poorer prognosis (Table 3).

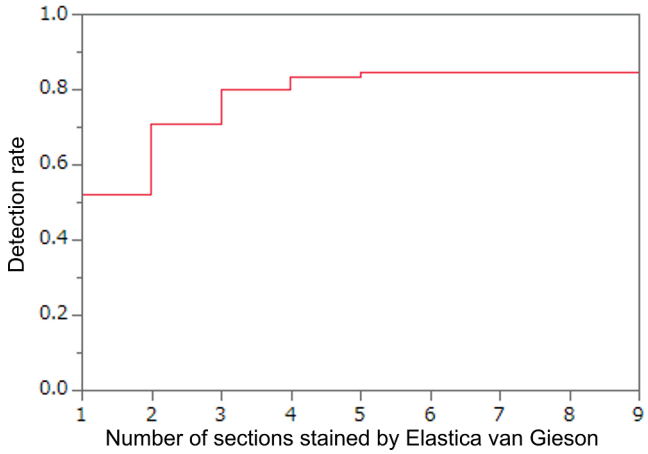

Fig. 2. Association between the number of slices of observed samples and the detection rate of venous invasion. The association between the number of slices and the detection rate of the HE-stained and EVG-stained samples was estimated by the Kaplan–Meier method. In the 1-, 2-, 3-, and 4-slice observations, the detection rates of venous invasion were 52, 71, 80, and 83.3%, respectively. For five slices and above, a plateau was reached, which was 84.7%.

3.4. Association between the number of sections subjected to EVG staining and the detection rate of venous invasion

The association between the detection rate of venous invasion and the number of samples was estimated using the Kaplan–Meier method (Fig. 2). When one slice of EVG was added (deepest HE + EVG), it was 52.0 %, and with two, three, four, and five or more slices, it was 71.0 %, 80.0 %, 83.3 %, and 84.7 %, respectively. No relationship was found between the number of slices of EVG-stained samples and the tumor diameter ($P = 0.5770$).

3.5. Association between venous invasion location and prognosis

For the deepest HE samples, no difference in the 5-year RFS was observed among the group without venous invasion, IMVI, and EMVI groups (Fig. 3A).

For both the deepest HE + EVG and whole-section HE, while a significant difference in the 5-year RFS was observed between the group without venous invasion and the EMVI group ($P = 0.030$ and $P = 0.0225$, respectively), no significant difference in prognoses was observed between the group without venous invasion and the IMVI group or between the IMVI and EMVI groups (Fig. 3B and C).

On the other hand, in the whole-section HE + EVG samples, significant differences in the 5-year RFS were observed between the absence group without venous invasion and the EMVI group ($P = 0.0027$). Despite no significant difference between the group without venous invasion and the IMVI group and between the IMVI and EMVI groups, a tendency was observed (Fig. 3D).

3.6. Interobserver concordance

The kappa values of the determination of venous invasion by deepest HE between the first dataset and that of the other two pathologists were 0.66 (95 % CI 0.50–0.84) and 0.66 (95 % CI 0.47–0.86) for SM and MN, respectively, showing substantial concordance.

The kappa values of the identification of venous invasion by deepest HE + EVG between the first dataset and that of the other two pathologists were 0.86 (95 % CI 0.76–0.96) and 0.84 (95 %CI 0.73–0.94) for SM and MN, respectively, showing almost perfect concordance.

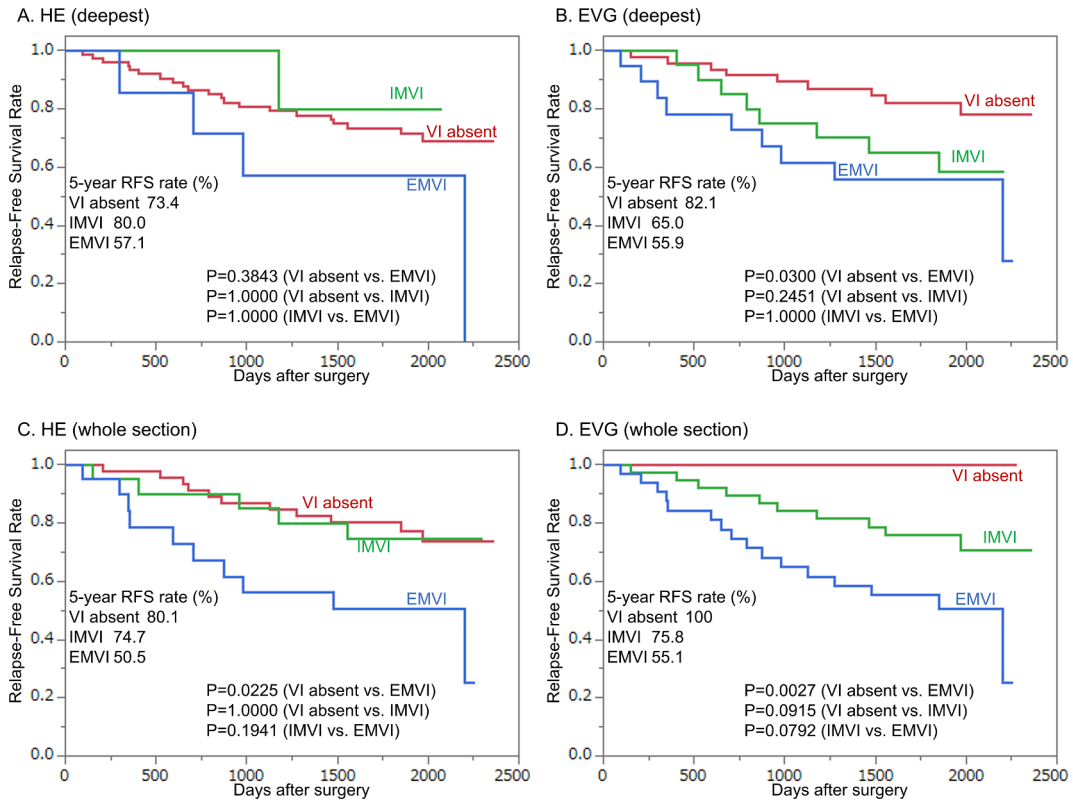

Fig. 3. Association between the position of the venous invasion and the relapse-free survival rate for each observation method. A, When observed with the deepest HE, no significant difference was found between the group without venous invasion and the EMVI group ($P = 0.3843$), the group without venous invasion and the IMVI group ($P = 1.0000$), and the IMVI and EMVI groups ($P = 1.0000$). B, When observed with the deepest HE + EVG, a significant difference was observed between the group without venous invasion and the EMVI group ($P = 0.0300$). No significant difference was observed between the group without venous invasion and the IMVI group ($P = 0.2451$) and between the IMVI and EMVI groups ($P = 1.0000$). C, When observed with whole-section HE, a significant difference was observed between the group without venous invasion and the EMVI group ($P = 0.0225$). There was no significant difference between the group without venous invasion and the IMVI group ($P = 1.0000$) and between the IMVI and EMVI groups ($P = 0.1941$). D, When observed with whole-section HE + EVG, a significant difference was observed between the group without venous invasion and the EMVI group ($P = 0.0027$). There was no statistically significant difference between the group without venous invasion and the IMVI group ($P = 0.0915$) and between the IMVI and EMVI groups ($P = 0.0792$), but there was a tendency. VI, venous invasion.

Table 4
Morphological characteristics of the cases in which venous invasion was determined using only HE-stained specimens.

Morphologic patterns	Present (%)
Orphanarteriole	48(98.0 %)
Protruding tongue	17(34.7 %)
Away from the main nodule	13(26.5 %)
Penetrating muscularis propria	5(10.2 %)

3.7. Morphological characteristics of the cases in which venous invasion was determined using HE-stained specimens only

(Table 4)Most venous invasions that could be determined with HE-stained specimens showed an ‘orphan arteriole’ (Fig. 4A and B) (48 cases: 98.0 %), a ‘protruding tongue’ pattern (Fig. 4C and D) (17 cases: 34.7 %), and a smooth, rounded nest away from the main nodule (Fig. 4F, and G) (13 cases: 26.5 %). The morphology ‘tumor lesions penetrate vertically in the gaps of the muscularis propria’ (Fig. 4H, I, and J) was found in only five cases (10.2 %). However, observation of HE-stained specimens confirmed venous invasion in all EVG-stained specimens at the site exhibiting the distinctive morphological feature. Conversely, venous invasion found in the main nodule was unrecognizable by HE staining alone (Fig. 4K and L).

4. Discussion

In this study, we examined the usefulness of EVG staining in determining venous invasion of colorectal cancer. We found that the addition of EVG staining to the conventional HE staining allowed the identification of venous invasion and improve the ability to predict patients’ prognoses.

Observations using elastin-stained specimens have been previously reported to improve the detection rate of venous invasion and the ability to predict patients’ prognoses [5,9–11]. However, another report suggested that additional specially prepared-stained samples were essentially useless because of the shift in the cross-section [2]. Some reports have indicated that careful assessment of morphological features such as ‘protruding tongue’ pattern and ‘orphan arteriole’ without using Elastica-stained specimens can yield results equivalent to those obtained using Elastica-stained specimens [13]. However, in our study, although we observed all tumor specimens with HE staining, we could not stratify patients by prognosis. In contrast, by adding EVG to just one slice (which included the deepest part of the tumor), the prognosis could be stratified. This result indicates that EVG staining is useful for evaluating venous invasion, which affects the prognosis. Furthermore, the venous invasion could be evaluated more accurately by increasing the number of EVG samples. Since the detection rate increased with each additional stained slice until the plateau in slices 4–5 was reached (Fig. 2), multiple slices of EVG-stained specimens were needed to conclude the absence of venous invasion. In our study, venous invasion identified by EVG staining was an independent factor indicating poor prognosis. Thus,

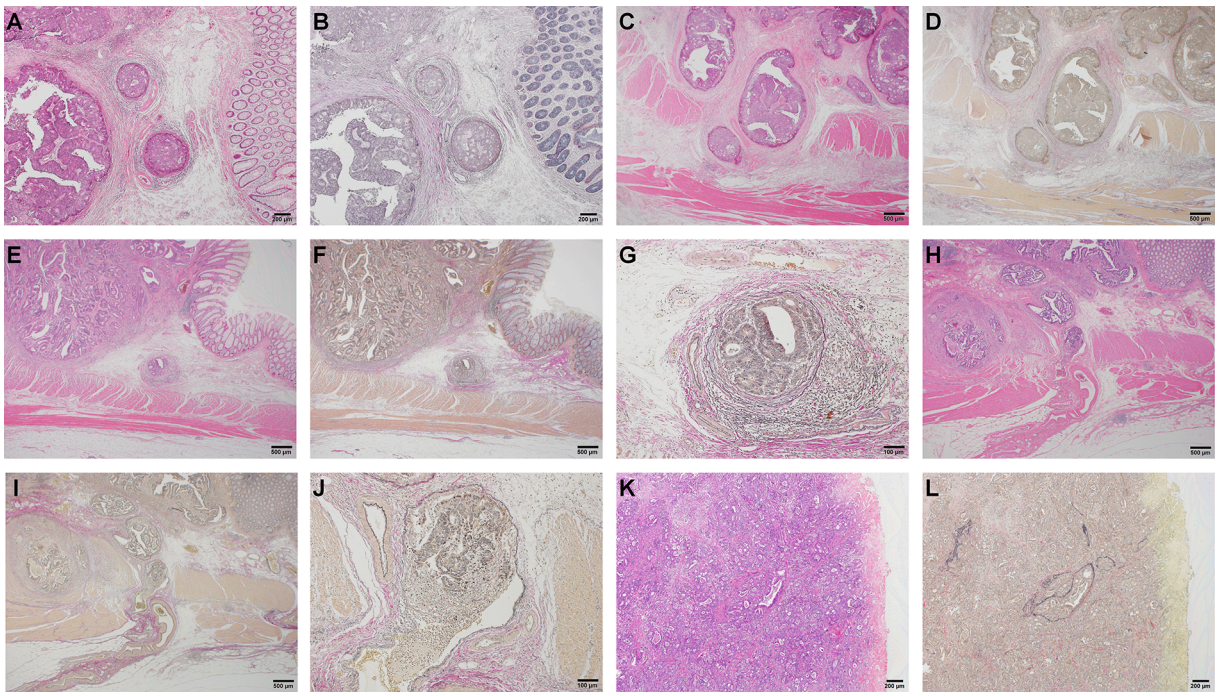

Fig. 4. Venous invasion (HE and EVG staining) with morphological features. A and B, 'orphan arteriole': A round tumor nodule with an adjacent artery. C and D, 'protruding tongue': Smooth tumor nodules on the tongue-protruding margin. In this image, the outer longitudinal muscle is preserved. E, F, and G, 'smooth, rounded nest, away from the main nodule': A round tumor nodule located away from the main tumor lesion. An artery is running next to it. H, I, and J, 'Penetrating muscularis propria': Tumor lesions penetrate vertically in the gaps of the muscularis propria.

venous specimens using EVG are indispensable for the diagnosis of colorectal cancer.

The actual relationship of IMVI with the prognosis remains debatable, and previous studies (albeit rectal studies) have shown that IMVI is barely associated with prognosis [2,15,16]. In our study, observation of whole-section HE-stained specimens showed no significant difference in RFS between the group without venous invasion and the IMVI group and between the IMVI and EMVI groups. However, the RFS rates in the group without venous invasion, IMVI, and EMVI groups tended to differ with respect to the observations of EVG staining of whole sections (Fig. 3). Based on whole-section observations using EVG staining, the prognosis of the three groups was considered stratified by accurate observation of IMVI and EMVI. Particularly for IMVI, many cases show thin vein walls and veins buried in tumors, making it difficult to identify them by HE staining alone.

Similar to previous studies, morphological features such as 'orphan arteriole' and 'protruding tongue' were useful for diagnosis in this study as well [9,12,13]. Although no reports have described the usefulness of the morphology 'tumor lesions penetrate vertically in the gaps of the muscularis propria', we found that focusing on this finding (Fig. 4H, I and J) made it easier to determine the venous invasion. The 'tumor lesions penetrate vertically in the gaps of the muscularis propria' refers to the findings at the site where the vasa recti, a branch of the marginal vein of the large intestine, penetrates the muscularis propria. The muscular layer of the penetrating part of the vasa recti is known to have a gap, which causes diverticulum of the large intestine. When the tumor lesions run vertically in the gaps of the muscularis propria, they often show venous invasion in the vasa recti. Of the 49 cases that could be considered as showing venous invasion by HE staining, only one (2 %) (Table 4) was diagnosed without recognition of the 'orphan arteriole'. In addition, the venous invasion amid the tumor was difficult to distinguish from the background tumor tissue (Fig. 4K and L).

By focusing on morphological features described in Table 4, inter-observer study of venous invasion identified by HE-stained only showed a high degree of agreement, but the addition of EVG staining further improved the agreement rate. These findings strongly support the usefulness of the morphological characteristics with venous invasion determined using HE-stained specimens only as well as the use of EVG staining in routine practice.

There are two possible reasons for the hesitance to perform EVG staining in daily diagnosis: First, EVG-stained specimen preparation is time-consuming, which delays the submission of pathology reports. Second, the preparation of EVG-stained samples involves an additional cost. However, if EVG staining is prepared on continuous sections simultaneously with HE specimen preparation, the time required for additional preparation can be avoided, and the submission of the pathology report would not be delayed. Regarding studies on the cost of preparing EVG-stained specimens, Abdulkader et al. [17] calculated an amount from the viewpoint of the number needed to treat in a study in which EVG staining was routinely performed for the pathological diagnosis of colorectal cancer. They determined that the additional cost to reduce one venous invasion-positive oversight was approximately £24.30. Therefore, they concluded that the cost was not substantial. Given these considerations, the additional time and cost required for routine EVG-stained specimen preparation do not seem to be as high as many pathologists believe [17].

This study had several limitations. First, it was a retrospective study conducted at a single facility. Second, the study was conducted using a relatively small number of cases. Third, risk factors other than a venous invasion that affect the prognosis, such as lymphatic invasion and desmoplastic reaction categorization, were not evaluated in this study.

5. Conclusion

In conclusion, we added EVG staining to the evaluation of colorectal cancer, which allowed the identification of venous invasion. Further increase in the number of EVG-stained specimens makes the diagnosis more accurate. For the evaluation of venous invasion for pT2–pT4 colorectal cancer, it is important to prepare specimens from multiple slices and multiple EVG stains to predict the prognosis.

Funding

No funding was obtained for this study.

Ethics statement including patient consent statement

The study was approved by the local ethics committee and conducted according to the principles of the Declaration of Helsinki. Consent was obtained from patients by giving them the right to opt out on the institutional website.

CRediT authorship contribution statement

Kota Nakashima: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization. **Jun Akiba: . Shinji Mizuochi:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Data curation. **Masamichi Nakayama:** Validation, Investigation. **Naohiro Yoshida:** Resources. **Kenichi Koushi:** Resources. **Takefumi Yoshida:** Resources. **Fumihiko Fujita:** Resources. **Hitoshi Obara:** Formal analysis. **Tatsuyuki Kakuma:** Formal analysis. **Yoshito Akagi:** Writing – review & editing. **Hirohisa Yano:** Writing – review & editing, Conceptualization, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Editage (www.editage.com) for English language editing.

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