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Original Article

Double immunohistochemical staining with laminin 5 (γ2 chain) and collagen IV in colorectal neoplasms

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Abstract: Colorectal cancer (CRC) is one of the most common cancer diagnoses in the Western world. It is outnumbered several times by the precursor stage adenoma. The aim of this study was to describe the expression pattern with a double immunohistochemical staining for laminin 5 (γ2) and collagen IV in different colorectal neoplasms. This might be a supplementary tool to morphology in diagnostic dilemmas as microinvasive pT1 tumors and adenomas with pseudoinvasion. Laminin 5 has been shown to stain in invasive tumor cells, while collagen IV highlights the basement membrane (BM). Fifty-seven patients divided according to the primary histopathological diagnoses of tubular adenoma, tubulovillous adenoma, adenoma with pseudoinvasion and glandular adenocarcinoma stages pT1, pT2 or pT3, were included in the study. In normal colonic mucosa, no expression of laminin 5 staining was observed. BM was always intact around normal crypts. In invasive tumors, laminin 5 stained intensely, and the BM was absent or focally discontinuous. The expression in adenomas and in pseudoinvasive areas was less consistent. The study suggests that double immunostaining with collagen IV and laminin 5 might be useful as a supplement for the diagnosis of pT1 CRC. In adenomas, the double staining highlights the areas for the pathologist to pay extra attention. By itself, the double staining cannot determine whether or not there is invasion. Morphology remains the single most important factor in differentiating adenoma and adenoma with pseudoinvasion from early invasive carcinoma.

Keywords: Colorectal cancer, colorectal adenoma, pseudoinvasion, multiple immunohistochemical staining, double immunolabelling

Introduction

Colorectal cancer (CRC) is one of the most common cancer diagnoses in the Western world, accounting for about 10% of all cancers. In 2012 the estimated number of new cases worldwide reached 1.3 mio [1]. This number is surpassed several times by the precursor stage, colorectal adenomas. According to the WHO criteria, the definition of CRC is invasion through the lamina muscularis mucosae as lesions with histopathological morphology of adenocarcinoma that are confined to the mucosa (in the literature sometimes called carcinoma in situ) have no risk of metastasis after complete removal by surgery [2]. A challenge in diagnostic biopsies or polypectomies is the presence of areas in an adenoma with pseudoinvasion, where colonic epithelium is misplaced into the submucosa below the level of muscularis mucosa [3, 4]. The displaced epithelium may harbor the same grade of neoplasia as does the surface epithelium of the adenoma. Often hemosiderin pigment, bleeding and dilated vessels are present and there may be cystic dilatation of glands with free mucin in the stroma. However, no desmoplasia of the surrounding stroma is present. Pseudoinvasion is reported in 2-10% of adenomas [3, 5] with the most frequent location being colon sigmoideum [3]. Differentiation of an adenoma with pseudoinvasion from an adenoma with a small area of invasive adenocarcinoma can be difficult, but have obvious important clinical implications, since pseudoinvasion is treated by polypectomy, whereas adenocarcinoma requires further diagnostic work-up, potentially further resection and a different follow-

Table 1. Number of patients in each category

Primary diagnosis	No. of patients
Tubular adenoma	10
Tubulovillous adenoma	10 (1 patient excluded, failure of immunohistochemical reaction)
Adenoma with pseudoinvasion	7 (1 patient excluded, the area with pseudoinvasion was missing on immunostained slides)
Adenocarcinoma, pT1	13
Adenocarcinoma, pT2	10 (1 patient excluded, too poor sectioning quality)
Adenocarcinoma, pT3	10

up [4]. Currently, the differentiation between these two entities relies solely on histopathological evaluation.

The basement membrane (BM) separates the epithelium from the underlying connective tissue in lamina propria. It regulates the organization of the epithelium and in addition has functions during cell proliferation, differentiation, adhesion and migration. Among other structural components, BM consists of collagen IV and laminin 5 [6-9] and a characteristic feature of the development of cancer is downregulation of collagen IV with resulting defects in the BM [4, 10-12].

Laminin is a heterotrimer composed of α, β and γ subunits of which more than 15 isoforms have been described [13]. Laminin 5 is composed of a α3, a β3 and a γ2 chain of which the γ2 chain is specific for laminin 5 [14-16]. Previous studies have shown that apart from being present in the BM, laminin 5 is also upregulated in the cytoplasm of invasive cancer cells [6, 14, 15] and it has been proposed as critical for cell migration and as a marker of invasiveness [17-19]. This is shown in several organs, including adenocarcinomas of the colon, breast and pancreas and squamous cell carcinoma of the skin, cervix and tongue [7, 11, 18-21]. Furthermore, it has been shown that in CRC the expression of laminin 5 is more intense in the invasive front and in areas characterized by cellular dedifferentiation [16, 20, 22, 23]. The expression of laminin 5 in the precursor stage adenoma is not as well examined and only few studies have been published with divergent results [15, 24].

In double or multiple immunohistochemical (IHC) staining procedures two or more targets is visualized simultaneously on the same slide. This method has some obvious advantages with the possibility of assessing several markers on the same slide, visualizing exactly the same cells [25]. Double IHC with laminin 5 and collagen IV has not previously been described in literature.

The primary aim of the present study was to describe the expression pattern of a double IHC staining with laminin 5 (γ2) and collagen IV in different colorectal neoplasms. If significant differences were seen this could possibly serve as a supplementary tool to morphology in distinguishing adenoma from carcinoma. It was hypothesized that invasive cells would stain intensively in laminin 5 (γ2) and a discontinuous BM around the invasive cells could be demonstrated by collagen IV.

Materials and methods

Patients

The study included 60 patients diagnosed at Bispebjerg Hospital and Rigshospitalet, Denmark, during 2014. The primary reported histopathological diagnoses were tubular adenoma (TA), tubulovillous adenoma (TVA), adenoma with pseudoinvasion or glandular adenocarcinoma stages pT1, pT2 or pT3. These categories were included in order to evaluate the expression pattern in the whole spectrum of colorectal neoplasms. **Table 1** shows the number of patients according to the primary diagnosis. Slides from all patients were retrieved from the archives and the diagnosis verified by two pathologists according to the 4th edition of the WHO classification. One slide being representative of the diagnosis was selected, and new sections of 4 μm thickness from the corresponding formalin-fixed and paraffin-embedded (FFPE) tissue block were cut for the following analysis. Three patients were excluded from the study leaving 57 patients for analysis.

Immunohistochemical staining

For all cases a slide was stained with hematoxylin and eosin, and both single and double IHC

Table 2. Tissue processing and staining protocol used

Step	Procedure	Reagent (vendor, cat. no.)	Time/ Temperature	Off/ On-board
1	Dewaxing and Heat Induced epitope retrieval	EnVision™ FLEX Target Retrieval Solution, Low pH (Dako, S1699)	20 min/97°C	Off
2	Rinse	Wash Buffer (Dako, S3006)	3×5 min/RT	Off
3	Mild proteolytic pre-treatment	Pepsin (Zytovision, ES-001-50)	5 min/RT	Off
4	Rinse	Wash Buffer (Dako, S3006)	3×5 min/RT	Off
5	Blocking of endogenous peroxidase activity	Peroxidase blocking solution (Dako, S2023)	5 min/RT	On
6	First Primary Antibody	Coll-IV clone CIV22 (1:25)	30 min/RT	On
7	Rinse	Wash buffer (Dako, S3006)	3×5 min/RT	On
8	Detection step 1	Quanto-Amplifier (Thermo S., TL-125-QPB)	10 min/RT	On
9	Rinse	Wash Buffer (Dako, S3006)	3×5 min/RT	On
10	Detection step 2	Quanto-HRP Polymer (Thermo S., TL-125-QPB)	10 min/RT	On
11	Rinse	Wash Buffer (Dako, S3006)	3×5 min/RT	On
12	Chromogen 1	Deep Space Black (Biocare, BRI4015H)	5 min/RT	On
13	Rinse	Wash Buffer (Dako, S3006)	3×5 min/RT	On
14	Second Primary Antibody	Laminin 5 (γ-chain) clone D4B5 (1:100)	30 min/RT	On
15	Rinse	Wash Buffer (Dako, S3006)	3×5 min/RT	On
16	Detection step 3	HiDef-Amplifier (Cell Marque, 962D-31)	10 min/RT	On
17	Rinse	Wash Buffer (Dako, S3006)	3×5 min/RT	On
18	Detection step 4	HiDef-AP Polymer reagent (Cell Marque, 962D-31)	10 min/RT	On
19	Rinse	Wash Buffer (Dako, S3006)	3×5 min/RT	On
20	Chromogen 2	Warp Red (Biocare, WR806H)	10 min/RT	On
21	Rinse	Tap water	2 min/RT	Off
22	Counter stain	Mayers Hematoxylin	3 min/RT	Off
23	Rinse	Tap water	3 min/RT	Off
24	Dehydration	99% Alcohol	3× (quick)/RT	Off
25	Clearing	Xylen	2×2 min/RT	Off
26	Permanent mounting	Pertex		

staining with laminin 5 (γ2) and collagen IV were performed. Single staining was performed in order to validate the double staining. The immuno-labeling experiments were performed using anti-human collagen IV clone CIV 22 (Dako, cat. no. M0785, Glostrup, Denmark) in combination with anti-human laminin 5 (γ2) clone D4B5 (EMD Millipore, cat. no. MAB19562, MA, USA). All incubation steps were automatically performed on the Autostainer LINK (Dako, Glostrup, Denmark) at room temperature (RT) and antigen retrieval procedures were done off-board. An overview of the double immunostaining technique is outlined in **Table 2**.

Briefly, dewaxing and antigen retrieval was performed by immersing slides in EnVision™ FLEX Target Retrieval Solution, Low pH (Dako, cat. no S1699) and heated in the PT-module for 20 min at 97°C, subsequently cooled to RT and placed in wash buffer (Dako, cat. no. S3006). Following heat induced epitope retrieval; slides were subjected to mild proteolytic pre-treatment using Pepsin solution (ZytoVision Gmbh, cat. no ES-0001-50, Bremerhaven, Germany) for 5 min at RT. After wash in buffer and blocking of endogenous peroxidise activity, slides were incubated with the collagen IV primary antibody (1:25) for 30 min at RT. Following the instructions given by the different manufacturers, the reactions for collagen IV were detected using the standard polymer technique Quanto-HRP (Thermo Scientific, cat. no. TL-125-QPB, MA, USA), and visualization was performed using Deep Space Black (Biocare Medical, cat. co. BRI4015H, Concord, USA). After the visualization with Deep Space Black, the slides were rinsed in wash buffer and subsequently incubated with laminin 5 (γ2) primary antibody (1:100) for 30 min at RT. The reactions for laminin 5 were detected with HiDef polymer-AP reagent (Cell Marque, cat. no. 962D-30, Rocklin, USA) and visualized with Warp Red alkaline phosphatase Substrate chromogen kit (Biocare Medical, cat. co. WR806H, Rocklin, USA). Finally, slides were rinsed in water, counterstained with Mayers haematoxylin and coverslipped.

The single staining were basically following the same procedures as for the double immunostaining except for collagen IV was used at dilution 1:50 and laminin 5 (γ2) was used at dilution 1:400. In addition, the reactions for each single primary antibody were detected using the standard polymer technique EnVision™ FLEX+/HRP Detection Reagents (Dako, cat. no. K8002) following the recommendations given by the manufacturer.

Positive controls with known expression of the respective antigens were included in the study: A colon specimen containing normal epithelium (for collagen IV) and a tumour area (for laminin 5 γ2). Negative controls were performed by omission of both primary antibodies, analyzing for potential unspecific staining of tissue components caused by the used detection systems.

No blocking steps was needed between the first and second set of immuno-reagents, using the advantages that DAB or DAB-based chromogens (Deep Space Black) efficiently blocks for cross-reactivity of the immuno-reagents involved in the second part of the double-immuno labeling process [26, 27].

Immunohistochemical evaluation

For all cases, the components normal colonic mucosa, adenoma, invasive tumor or areas with pseudoinvasion was evaluated separately when present. In cases of adenomas with both low and high grade neoplasia, only areas with high grade neoplasia were assessed. The cytoplasmic staining intensity of laminin 5 (negative, weak, moderate or intense staining) in epithelial cells was registered. Focal staining was sufficient for a case to be registered as positive. At least two tubular structures of five tumor cells or a corresponding number of single cells where required for a case to be registered as positive. For invasive tumors, it was also assessed if the laminin 5 (γ2) expression was more intense in the invasive tumor front than in the rest of the tumor, and if lower differentiated parts including tumor buds stained more intensely compared to glandular areas. The invasive front was defined as a region of 500 μm width at the tumor periphery. A tumor bud was defined as a cluster of one to four tumor cells, detached from the invasive border.

The BM surrounding crypts or epithelial cells were registered as being complete or incomplete by assessment of the collagen IV expression. A strong continuous staining of the BM surrounding the epithelium was registered as complete, whereas an absent or discontinuous BM was assessed as incomplete. In cases with an incomplete BM, it was noted if the location of the epithelial cells in question was above or beneath the lamina muscularis mucosae and if this was found in relation to areas with intense laminin 5 (γ2) expression.

All cases were evaluated independently by two pathologists. Discrepant cases were reviewed by a third pathologist and a consensus judgment was reached.

Statistics

Comparison of staining intensities in the three compartments was statistically evaluated by a two-sided Fisher's exact test. *P*-values lower than 0.05 were considered statistically significant. For the statistical analyses, no or weak staining with laminin 5 (γ2) was considered a negative reaction. Moderate or intense staining was considered a positive reaction. The statistical analyses were calculated using GraphPad QuickCalc's Web site http://www.graphpad.com/quickcalcs (accessed August 2016).

Ethics

The study was approved by the Local Committee on Health Research Ethics on permission number H-9-2014-012 and by the Danish Data Protection Agency on permission number REG-51-2015.

Results

Out of a total of 570 registrations in 23 (4%) cases, the two pathologists had categorized the cases differently and a consensus diagnosis was needed. The results are shown in **Table 3**.

Normal colonic mucosa

In 47 cases normal mucosa was represented. In one case of a TVA laminin 5 (γ2) stained weak in normal colon epithelium while in the remaining cases no expression was seen, thus all cases was considered as negative for the

Table 3. Results of double immune staining with collagen IV and laminin 5 (γ2)

	pT1 N=13	pT2 N=9	pT3 N=10	TA N=10	TVA N=9	Pseudoinv N=6
Laminin-5 intensity	Negative: 12	Negative: 8	Negative: 4	Negative: 10	Negative: 6	Negative: 6
Normal mucosa	No representation: 1	No representation: 1	No representation: 6		Weak: 1	
					No representation: 2	
Laminin-5 intensity	Weak: 1	Moderate: 1	Intense: 1	Weak: 1	Moderate: 4	Moderate: 2
Adenoma component	Moderate: 4	Intense: 1	No representation: 9	Moderate: 5	Intense: 5	Intense: 4
	Intense: 4	No representation: 7		Intense: 4		
	No representation: 4					
Laminin-5 intensity	Intense: 13	Intense: 9	Intense: 10	No representation: 10	No representation: 9	Weak: 1
Invasive/pseudoinvasive area						Moderate: 1
						Intense: 4
BM complete or incomplete	Complete: 12	Complete: 8	Complete: 4	Complete: 10	Complete: 7	Complete: 6
Normal mucosa	No representation: 1	No representation: 1	No representation: 6		No representation: 2	
BM complete or incomplete	Complete: 5	Complete: 2	Complete: 1	Complete: 4	Complete: 4	Complete: 5
Adenoma component	Incomplete: 4	No representation: 7	No representation: 9	Incomplete: 6	Incomplete: 5	Incomplete: 1
	No representation: 4					
BM complete or incomplete	Incomplete: 13	Incomplete: 9	Incomplete: 10	Not relevant: 10	Not relevant: 9	Complete: 3
Invasive/pseudoinvasive area						Incomplete: 3
More intense laminin-5 in tumorbuds or lower differentiated parts of tumor?	Yes: 12	Yes: 9	Yes: 10	Not relevant: 10	Not relevant: 9	Not relevant: 6
	No lower differentiated areas: 1					
More intense laminin in invasive front?	Yes: 11	Yes: 5	Yes: 9	Not relevant: 10	Not relevant: 9	Not relevant: 6
	No: 2	No: 4	No: 1			
Does loss of collagen IV correlate with intense expression of laminin-5?	Yes: 13	Yes: 9	Yes: 10	Yes: 6	Yes: 5	Yes: 2
				Not relevant: 4	Not relevant: 4	Not relevant: 4
Location of incomplete BM and intense laminin	Submucosa: 13	Submucosa: 9	Submucosa: 10	Lamina propria: 6	Lamina propria: 5	Lamina propria: 2
				Not relevant: 4	Not relevant: 4	Not relevant: 4

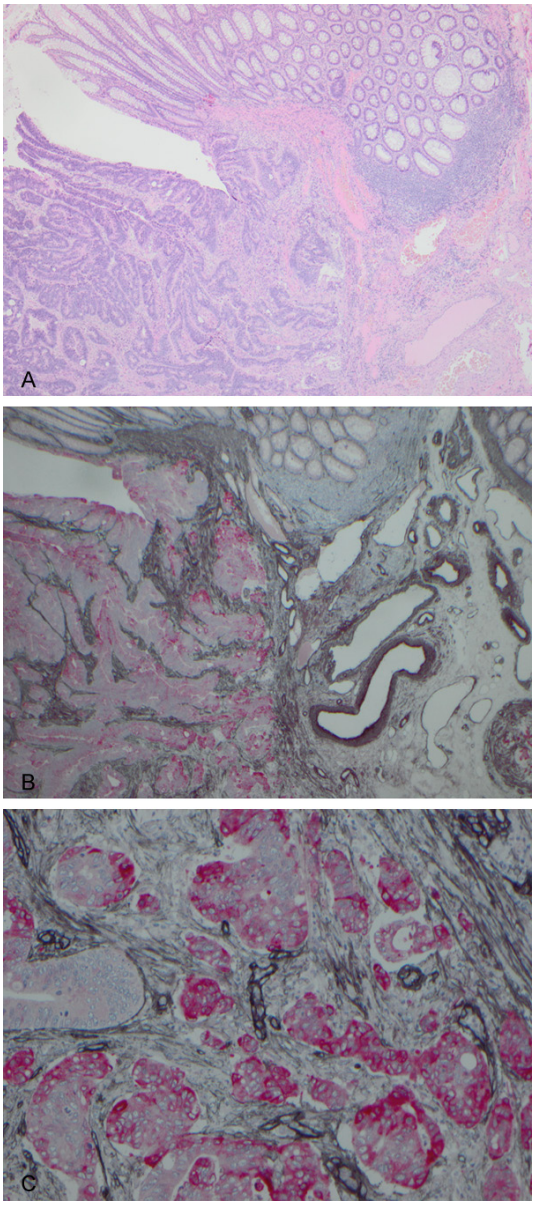

Figure 1. A: HE, ×40 magnification. Normal colonic mucosa is seen in upper right corner. Invasive tumor (pT1) on the left side. The invasive margin shows lower differentiation. B: Laminin 5 (γ2)/collagen IV double immunostaining of the same tumor as in A, ×40 magnification. Laminin 5 (γ2) stains red in the cytoplasm of the tumor cells, while no expression is seen in normal mucosa. Collagen IV stains the basal membranes and is seen as an intact black line surrounding the normal crypts, whereas the basal membranes are disrupted in the invasive part of tumor. C: Laminin 5 (γ2)/collagen IV, ×100 magnification. The photo shows the invasive margin of the tumor in A and B. The laminin 5 (γ2) staining is intense in the lower differentiated parts of tumor.

statistical analyses. BM was in all cases continuously stained around normal crypts with collagen IV (**Figure 1A-C**).

Adenomas

In 19 adenomas the expression of laminin 5 (γ2) in one case was considered as negative while the remaining 18 cases were considered as positive. No difference between TA and TVA was observed. In six adenomas with pseudoinvasion the adenoma compartment was positive in all cases. In 12 cases of invasive tumors, an adenoma component was also present, of which one was negative and 11 positive. In 11 adenomas out of 19, the collagen IV staining of BM was incomplete while this number was one out of six in the adenoma compartment in cases with pseudoinvasion and four out of 12 in the adenoma compartment of the invasive tumors. The discontinuous BM was always being located above the lamina muscularis mucosae thus corresponding to carcinoma in situ.

Carcinomas

Laminin 5 (γ2) was positive in all invasive tumors, i.e. 32 cases. The reaction was observed to be more intense in lower differentiated parts, tumor buds and in single tumor cells compared to the rest of the tumor. In one tumor, however, only a highly differentiated area was present, and thus this parameter could not be accessed. In 25 (78%) cases, the reaction was more intense in the invasive front. In all cases of invasive tumor, the collagen IV staining of BM was focally incomplete or absent and the localization always corresponded to an area with more intense laminin 5 (γ2) expression of the neoplastic cells (**Figure 1A-C**).

Pseudoinvasive areas

In the six cases with pseudoinvasion, this area was negative for laminin 5 (γ2) in one case and positive in five cases. Collagen IV staining of BM was complete in three cases and incomplete in three cases.

Overall, it was noticed that in highly differentiated parts of a carcinoma the BM occasionally displayed intact expression for collagen IV in areas that was obviously invasive. It was also registered that in areas with inflammation or crypt abscesses, laminin 5 (γ2) expression of the epithelial cells was intense with disrupted collagen IV staining in the BM. This was observed not only in invasive parts of a tumor but also in adenomas.

Statistical analyses

Comparing normal mucosa vs. invasive tumor and normal mucosa vs. adenoma showed statistically significant differences in staining pattern of both laminin 5 (γ2) and collagen IV ($P<0.0001$). Statistically significant differences was also found comparing the expression pattern of collagen IV in adenoma vs. invasive tumor ($P<0.0001$), whereas no statistically difference was observed for laminin 5 (γ2) expression. The calculated *p*-values were significant, regardless of whether only the adenomas (no: 19) or also the adenoma compartment represented in invasive tumors and adenomas with pseudoinvasion (no: 37) were included in the calculations. The number of adenomas with pseudoinvasion was too small for statistical analyses.

Discussion

The present study showed that in normal colonic mucosa the BM (collagen IV) was always intact and no expression of laminin 5 (γ2) of normal epithelial cells was seen. Conversely, in all carcinomas an absent or discontinuous BM was seen accompanied by increased expression of laminin 5 (γ2) in the tumor cells. The reaction was more pronounced in poorly differentiated areas, in tumor buds and in the invasive margin. In adenomas and adenomas with pseudoinvasion the differences were less clear. A discontinuous BM was observed in about half of the cases with correspondingly increased expression of laminin 5 (γ2). This was seen in areas where the crypts exhibited signs of degeneration with leucocyte infiltration and rupture i.e. discontinuation in the epithelial cells lining the crypt and in areas with high grade neoplasia. The location was always above the lamina muscularis mucosae corresponding to adenocarcinoma in situ.

Although loss of a continuous BM is described as associated with increasing malignancy, loss of continuity of BM has also previously been described in some benign lesions as for example inflammatory diseases and, conversely, a complete BM can be present in invasive tumors [6, 8, 12]. This correlates well with the finding in this study where an incomplete BM was observed in areas with inflammation and a complete BM was seen in higher differentiated areas of carcinomas. This means that visualizing the BM by use of immunostaining for collagen IV is not an absolute criterion for invasiveness.

The expression of collagen IV has been investigated in normal mucosa, adenoma, and CRC. In one previous study, the authors report an intact BM surrounding not only normal crypt epithelium but also all of the adenomas no matter type and grade of dysplasia [12]. In contrast to this, Radovic et al. described significant changes in expression of collagen IV in both inflammatory and neoplastic lesions of colon. More prominent changes were observed to be associated with increasing neoplastic potential [28]. In our study, an incomplete BM was observed in about half of the adenomas and discontinuation was localized in areas with inflammation or high grade neoplasia, sometimes in areas of carcinoma in situ. Areas with defects in BM correlated with strong expression of laminin 5 (γ2).

A study by Yantiss et al. has evaluated the utility of collagen IV among other IHC stainings in differentiating adenoma with pseudoinvasion from adenocarcinoma [4]. The results are more consistent compared to ours. They found a continuous BM in 100% of 23 patients with pseudoinvasion while the BM was discontinuous in 96% of 23 patients with adenocarcinoma. The authors described that cases of pseudoinvasion are often associated with dilated, ruptured crypts with extravasated mucin and inflammation. In the present study in these areas an incomplete BM and strong expression of laminin 5 (γ2) was seen. Thus, although the number of cases with pseudoinvasion was small in our study this alone cannot explain the difference.

Several studies have evaluated the expression of laminin 5 in colorectal adenomas and CRC. Direct comparison is difficult because the methods are different and the definitions of a positive vs. a negative reaction are not similar. Some studies use the percentage of positive cells varying from 5% to 30% for a tumor to be reported as positive [15, 16, 23, 29]. Others use the relative number of positive cells and staining intensity and correlate this to a graded score [14, 18, 20, 22].

In this study the expression of laminin 5 (γ2) was scored as no staining, weak, moderate or intense staining with focal expression being sufficient. This estimate was thought to be

reproducible. For the statistical analyses, no and weak staining was pooled in a negative group, while moderate and intense staining made up a positive group. Only few cases stained weakly, and it did not affect the results if the weakly staining cases were pooled together with the moderately and intensely in a positive group.

All of the carcinomas expressed laminin 5 (γ2). This is in accordance with some of the previous observations [18, 20, 22], which report increased expression in 89-100% of CRCs. Also in squamous cancer, i.e. in cervix and the tongue, a similar expression level is reported [7, 19]. In contrast, a study by Masaki et al. reported that only 30% of the evaluated pT1 tumors were positive [23]. This might be explained by a higher cut-off value of >30% positive cancer cells for a case to be registered as positive. Another study reported 1/3 to 2/3 of tumors to be laminin 5 positive. Again higher cut-off values were used [29].

In all of the cancer cases, the staining was heterogeneous with a gradient towards a more intense staining in lower differentiated areas and tumor buds, and in a high number of the tumors a more intense staining was also observed in the invasive margin. This also correlates with previous reports [7, 14, 18, 20-22]. Since laminin 5 has been reported to have cell migration promoting activity, the lower differentiated areas may indicate high migratory capacity [17]. Conversely, no expression of laminin 5 (γ2) was seen in normal mucosa. This is consistent with what was found by several other authors [7, 14, 18, 21, 22]. In contrast it has also been described that staining is seen in most BM of normal mucosa [30]. This seems reasonable, since laminin 5 is one of the components of the BM. A study by Lohi et al. described reactivity for laminin 5 by use of immunofluorescence in BM of adenomas as well as carcinomas [9]. Sordat et al. has examined the expression of laminin 5 subunits in colorectal neoplasia. Again by use of immunofluorescence, they demonstrated expression in BM of both normal colonic mucosa and adenomas while a cytoplasmic reaction was only seen in the invasive budding cells [24]. The explanation of this divergence may be different visualization techniques as well as different sensitivity of the antibodies. Importantly, in the present study the reactions for laminin 5 (γ2) cannot be identified in BM due to the strong sheltering capacity of the DAB based chromogen Deep Space Black (collagen IV) [26, 27].

Lenander et al. have shown that the expression of laminin 5 in adenomas increases progressively towards a more atypical phenotype and is thus an indicator of early invasiveness [15]. This previous study included both hyperplastic polyps with no malignant potential and tubular, tubulovillous and serrated adenomas with a malignant potential. In this study, TA and TVA and adenomas in continuity with a carcinoma was included. No difference among the subgroups of TA vs. TVA was seen. In most cases, a component of high grade neoplasia was present and only this part of the adenoma was scored. In total, only in two cases the adenoma compartment stained weakly while in the remaining cases moderate or intense staining was observed. The reported higher rates of laminin 5 (γ2) staining in the present study and the lack of difference between the two groups might suggest that the grade of neoplasia is more important than the adenoma subgroup.

A correlation between a poor prognosis and increased laminin 5 expression in colorectal adenocarcinomas has been proposed [14, 16, 22, 23]. We did not register clinical information of the patients, but since all stages of the included invasive tumors showed intense expression of laminin 5, it would not have demonstrated a correlation to prognosis.

Conclusively, the double IHC with collagen IV and laminin 5 show significant differences in expression patterns in colorectal neoplasms and might be used as a supplement for the diagnosis of pT1 tumors. Furthermore the staining helps in highlighting the area where the pathologist has to pay extra attention, but the morphology remains to be the single most important factor in differentiating adenoma and adenoma with pseudoinvasion from early invasive carcinoma.

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Disclosure of conflict of interest

None.

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