

Expression of the EGFR-RAS Inhibitory Proteins DOK1 and MTMR7 and its Significance in Colorectal Adenoma and Adenoma Recurrence

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ABSTRACT

Background & Aims: Colorectal adenomas are precursor lesions for colorectal cancer (CRC), a major cause of cancer-related death. Despite all molecular insights, there are still unknown variables in the development of CRC as well as uncertainties regarding adenoma recurrence after resection. We aimed to characterize the expression of docking protein 1 (DOK1) and myotubularin-related protein 7 (MTMR7), which share inhibiting functions on EGFR-RAS-signalling, a major oncogenic driver in CRC, and their association with clinical variables and adenoma recurrence.

Methods: This observational study is based on clinical data obtained from patients who underwent routine endoscopy and consecutive follow-up examinations. Immunohistochemistry was conducted both in dysplastic tissue and adjacent non-dysplastic mucosa followed by microscopical assessment. Recurrence was differentiated between local, segmental and distant relapse.

Results: A total of 56 patients (23 females) gathering 96 adenomas/polyps were included. 36 patients experienced a metachronous lesion, 23 patients had simultaneous lesions in their index endoscopy. Female patients showed lower levels of MTMR7 in adenomas ($p=0.0318$). Adenomas of young patients showed lower DOK1 than those of older patients ($p=0.0469$). Big adenomas showed a higher expression of DOK1 than small lesions ($p=0.0044$). In serrated lesions, DOK1 was reduced ($p=0.0026$) and correlated with the quantity of lesions ($p < 0.001$). MTMR7 was significantly reduced in distant ($p=0.05$) and local segmental recurrence ($p=0.0362$), while DOK1 showed higher expression in recurrence ($p=0.0291$).

Conclusions: We found ambivalent results regarding the role of the markers as potential tumor suppressors, implying a context-dependent function of these molecules which might change in the course of time. DOK1 may play an inhibiting role in the serrated pathway. Remarkably, molecular markers have the potential to predict recurrence, since a combined expression analysis of high DOK1 and low MTMR7 correlated with the likelihood of segmental adenoma recurrence.

Key words: colorectal adenoma – adenoma recurrence – DOK1 – MTMR7 – serrated adenoma.

Abbreviations: AKT: thymoma viral proto-oncogene; APC: adenomatous polyposis coli; AUC: area under curve; BRAF: v-raf murine sarcoma viral oncogene homolog B1; CDX2: caudal-related homeobox 2; CI: confidence interval; CRC: colorectal cancer; CIMP: CpG island methylator phenotype; DOK1: docking protein 1; EGFR: epidermal growth factor receptor; ERK: extracellular signal-regulated kinase; GAP: GTPase-activating protein; KRAS: Kirsten rat sarcoma virus; MTMR7: myotubularin-related protein 7; NAT: normal mucosa adjacent to tumor; PIP3: phosphatidylinositol 3-phosphate; RAS: rat sarcoma virus; SD: standard error; SL: serrated lesions; TA: traditional adenomas.

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INTRODUCTION

Despite continuous medical advancements, colorectal cancer (CRC) remains one of the leading causes of cancer death worldwide [1]. Colorectal adenomas are widely accepted as precursor

lesions of CRC, and the adenoma-carcinoma sequence involves a series of mutational events. Alongside molecular risk factors of adenoma development such as adenomatous polyposis coli (APC) and Kirsten rat sarcoma virus (KRAS) gene mutations, recent research characterized additional clinical risk factors such as age, smoking and other behavioural factors [2].

Colorectal adenomas can be categorized into two main groups: the traditional adenomas (TA) (comprising tubular,

tubulovillous and villous adenomas) and the serrated lesions (SL) (comprising hyperplastic polyps, sessile serrated adenoma/polyps and traditional serrated adenomas). The different histological subtypes arise from different molecular patterns and mutations. Traditional adenomas and SL have similar mutation frequencies but different driver mutations and epigenetic characteristics. While TA are mostly characterized by APC mutations [3], key features of the serrated pathway are v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutations and CpG island methylator phenotype (CIMP) [3, 4].

Screening endoscopy as secondary prevention has proved to increase survival and reduce CRC-related mortality [5, 6], but acceptance remains rather poor. In addition, the recommendations for the follow-up examinations vary in the different guidelines and are more based on the expert opinions than on the medical evidence.

Common risk factors for the recurrence of polyps and adenomas are polyp size, resection technique and incomplete resection [7, 8]. In recent years, there also were some papers on the molecular characterisation of adenoma recurrence. For instance, a gain of caudal-related homeobox 2 (*CDX2*) was identified as a predictor of recurrence [9]. But still, little is known about molecular risk factors of recurrence. Thus, a better assessment of the need of follow-up endoscopies could reduce the load of examinations and improve acceptance in patients.

The tumor suppressors docking protein 1 (*DOK1*) and myotubularin-related protein 7 (*MTMR7*) were previously characterized as interfering with downstream EGFR-RAS-signalling (EGFR: epidermal growth factor receptor; RAS: rat sarcoma virus;) in the context of CRC. Primarily, *DOK1* was well investigated in the haematological disorders such as Burkitt's lymphoma [10] and in the solid tumors such as lung cancer [11]. Its molecular function as versatile docking protein enables *DOK1* to inhibit ERK1/2 signalling downstream the EGFR signal cascade by binding the p120 RAS GTPase-activating protein (GAP) [12, 13]. Recent research also revealed growth-inhibiting features next to prognostic value in CRC [14]. By its function as a phosphatidylinositol 3-phosphate (PIP3)-phosphatase, *MTMR7* can inhibit insulin-mediated AKT-ERK1/2 (AKT: thymoma viral proto-oncogene; ERK: extracellular signal-regulated kinase) signalling-dependent growth in CRC cell lines. In addition, its abundance is inversely correlated with the tumor stage in CRC [15].

Since the molecular risk factors could help to further stratify the risk of the adenoma recurrence, we asked whether the loss of these tumor suppressors would affect the risk of relapse. In addition, there is little knowledge when loss of these molecules during progression of precursor lesions occurs.

Therefore, the aim of this study was to find out when these potential tumor suppressors are down-regulated in the adenoma-carcinoma sequence, if a loss of these markers is a risk factor for recurrence of colorectal adenomas and if there are differences in their expression between subtypes of adenomas.

METHODS

Data Collection

Patients who underwent resection of the colorectal adenomas at the Central Interdisciplinary Endoscopy Unit of

Mannheim University Hospital between 2004 and 2011 with consecutive follow-up endoscopies and provided an informed consent were included (approved by Local Ethics Committee, Medizinische Ethikkommission II, Heidelberg University, identifier: 2016-541N-MA). Localization of the adenoma was subdivided according to the anatomical structures into rectum, sigma, colon descendens, splenic flexure, colon transversum, right colon flexure, colon ascendens and caecum. Dysplasia of adenomas was determined according to the Vienna classification. Clinical data of patients including the polyp characteristics such as size, location, histopathological classification, recurrence or metachronous colorectal adenocarcinoma were retrieved from the electronic database of the Central Interdisciplinary Endoscopy Unit of the Mannheim University Hospital.

The procedure of polypectomy depended on the size and localisation. Snare polypectomy, endoscopic mucosal resection and endoscopic submucosal dissection comprised the techniques applied. Follow-up endoscopies were conducted either at the University Hospital Mannheim or with collaborating gastroenterologists; time to follow-up depended on the histological subtype and recommendations given by the current clinical guidelines [16].

Since the patients' endoscopically obtained tissue comprised dysplastic and adjacent non-dysplastic, but no distant normal colon tissue, we additionally conducted immunohistochemistry of normal colon tissue arrays.

Tissue Processing, Immunohistochemistry and Microscopical Scoring

After resection, the samples were processed to formalin-fixed, paraffin-embedded tissue and subsequent haematoxylin & eosin staining according to standard operating procedures of the Institute of Pathology of the University Hospital Mannheim. Thereafter, tissue sections were examined and classified by experienced pathologists. For immunohistochemistry analysis, we used a two-step protocol with rabbit anti-human primary antibodies and a biotinylated horse-radish peroxidase-labelled goat anti-rabbit secondary antibody. Formalin-fixed, paraffin-embedded tissue was dissected on a microtome into 3µm sections. Heat-mediated antigen retrieval with low pH, citric acid – based unmasking solution (Vector Laboratories, Burlingame, California) was performed for 15 (*MTMR7*) or 20 minutes (*DOK1*) following a peroxidase block with 3% hydrogen peroxide solution (Merck, Darmstadt, Germany) for 20 minutes to quench endogenous tissue peroxidase. Prior to incubation with primary antibodies, a blocking step using a solution of 5% goat serum dissolved in 1% bovine serum albumin (BSA)/phosphate-buffers saline (PBS, Merck) was conducted for 1 hour. Anti-human *DOK1* antibody (Abcam, Cambridge, United Kingdom) was diluted 1:100 in 5% goat/1% BSA staining buffer. Anti-human *MTMR7* (MyBiosource, San Diego, California) was diluted 1:200 in the same staining buffer. After incubation overnight at 4°C, the avidin/biotin-based ABC detection kit (Vector Laboratories) containing a horse-radish-labelled secondary antibody and 3,3'-diamino benzidine (DAB) brown colour substrate was used for antigen detection following the manufacturer's protocol (see

supplementary table 1 for additional information). The same staining methods were applied to tissue microarrays of normal colon tissue (CO727, US Biomax, Rockville, Maryland).

Stained tissue underwent digital bright-field microscopy (Leica Application Suite, Leica, Wetzlar, Germany). To quantify marker expression in dysplastic and adjacent non-dysplastic tissue, we applied an adjusted immunohistochemical score (H score) as previously described [17]. In brief, the percentage of stained cells (0=<25%, 1=25-50%, 2=50-75%, 3=75-100%) and staining intensity (0=no staining, 1=weak staining, 2=moderate staining, 3=strong staining) were multiplied to calculate H scores, resulting in a range from 0 to 9 (Fig. 1).

Classification and Statistics

We compared expression in different histopathological subtypes (TA & SL). Recurrence of lesions was subdivided into local recurrence (meaning recurrence exactly in the area of the prior lesion) recurrence within the same colonic segment or metachronous lesions in distant segments. Since the incomplete resection is an independent risk factor of recurrence, we only included adenomas with a known status of complete resection.

H scores result in ordinal scale data, so we conducted Mann – Whitney *U* test for univariant comparison with GraphPad Prism (GraphPad Software, San Diego, California). To calculate multivariate statistics, odds ratios and logistic regression we used SAS software (SAS Institute, Cary, North Carolina). For prediction of relapse with logistic regression, we used a stepwise selection model of combined expression results. Fisher's exact test was conducted for contingency of expression and clinical variables (GraphPad Prism). To gain more detailed insights of expression effects, we chose two different approaches to analyse the adenoma expression data. In an adenoma-related analysis, we examined expression of DOK1/MTMR7 and its relation to clinical factors in a single polyp-specific manner. For a case-related analysis, we combined the scores of the simultaneously removed adenomas of every individual patient. This allowed us to draw organ-wide conclusions in contrast to adenoma-related analysis. Significance level was set to 0.05 (5%), confidence interval was set to 95%.

RESULTS

A total of 56 patients (23 females, 33 males) gathering 96 polyps were included in this study. Mean age at polypectomy was 62.5 years [median 62 years, standard deviation (SD) 10.79 years]. Of those patients, 36 had one or more simultaneous lesions, 20 had none. In total, 20 patients developed a metachronous polyp/adenoma, 36 patients did not. The group of SL comprised of 42, the TA group of 48, a number of 6 adenomas were either a mixture of histopathological subtypes or could not be classified. No patient was diagnosed with CRC on the follow-up examinations. Localization for 90 polyps was specified, and distribution between the left and the right colon was equally balanced (43 polyps in the right colon, 47 in the left colon). The majority of patients had simultaneous lesions (35 patients with > 1 polyp, up to 7 simultaneous lesions, median 1, SD 1.67). Clinical variables are summarized in Table I.

Correlation of Patients' Characteristics with Expression of DOK1 and MTMR7

The expression of MTMR7 and DOK1 revealed differences regarding the age of patients. Divided into a group of old (>62 years) and young (<62 years) patients by the median age at polypectomy, MTMR7 showed a tendency for a lower expression in non-dysplastic tissue of older patients (old vs. young subgroups; median H score: 1.00, SD: 1.71; 2.00, SD: 2.35; $p=0.0544$). DOK1 showed a lower expression in dysplastic tissue of young patients (old vs. young subgroups; median H score: 3.65, SD: 1.64; 2.29, SD: 2.23, $p=0.0469$) (Fig. 2 and Table II). Dependency of age and expression of DOK1 was confirmed in a chi-squared test ($p=0.049$).

The analysis of dysplastic tissue of 55 polyps from male patients and 41 from female patients showed a higher expression of MTMR7 in the male patients (male vs. female subgroups; median H score: 1.75, SD: 1.20; 1.20, SD: 1.18; $p=0.0318$). A chi-squared test confirmed interrelationship of gender and MTMR7 expression ($p=0.0258$) (Fig. 3). DOK1 showed no gender-depending differences in protein expression.

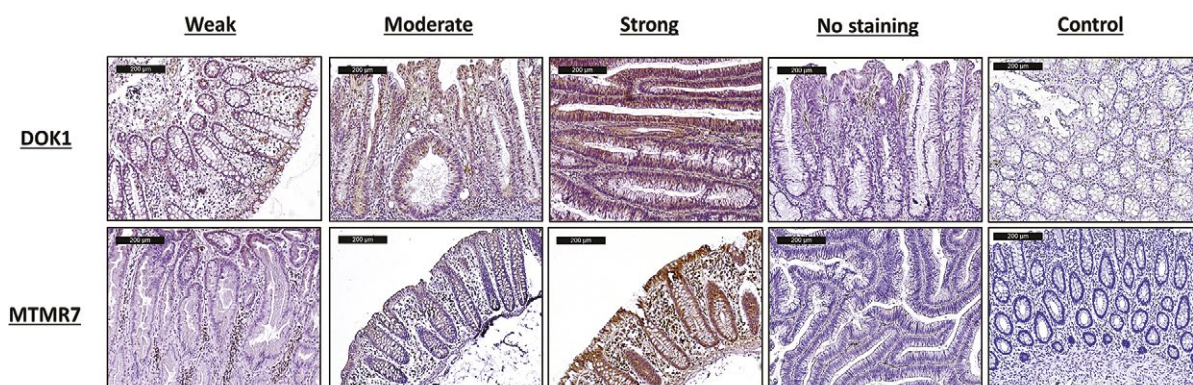


Fig. 1. Exemplary images for the applied staining intensities in epithelial cells and appropriate negative controls (control) for all examined markers, 200x magnification bright field microscopy. Tissues harbour intrinsic positive controls (lamina propria cells) as can be observed in “no staining” exemplary pictures in comparison to “control” pictures.

Table I. Overview of clinical characteristics

Characteristic	
Number of patients	56
Number of polyps/adenomas	96
Median age at endoscopy (years)	62 (SD 10.79)
Sex distribution	
Female	23
Simultaneous lesions	
Yes	36
No	29
Relapse	
Yes	20
No	36
Localization	
Left	47
Right	43
Undetermined	6
Histology	
Traditional adenomas	48
Serrated lesions	42
Undetermined/mixture	6
Grade of neoplasia	
Low-grade	56
High-grade	32
Undetermined	8

SD: standard deviation. Histology, grading of neoplasia or localization were partly undetermined. These cases were ruled out for particular statistics.

Expression of DOK1 & MTMR7 and Correlation to Dysplasia, Adenoma Size and Simultaneous Lesions

The evaluation of expression profiles revealed a lower protein expression of DOK1 in dysplastic tissue than in adjacent non-dysplastic tissue (dysplastic vs. non-dysplastic groups; median H score: 3.40, SD: 2.07; 4.00, SD: 2.30, $p=0.0087$). For MTMR7, no difference was found between dysplastic and adjacent non-dysplastic tissue (Fig. 2, Supplementary file). In addition, the staining of normal colon tissue arrays revealed the

expression of both DOK1 and MTMR7 in the colon mucosa of the healthy controls as well as in the tumor adjacent mucosa (Supplementary file). Neither DOK1 nor MTMR7 showed any difference in protein expression between high-grade or low-grade neoplasia.

Size in terms of diameter was known of 90 polyps. The binary classification into big or small varies in international classification. While the German guideline on colorectal cancer and adenomas [16] applies a 10 mm cut-off for big vs. small, other publications use a 20 mm cut-off [2]. We applied the 20 mm cut-off margin and found a higher expression of DOK1 in dysplastic tissue in big adenomas compared to small adenomas (dysplastic tissue, big adenomas vs. small adenomas, mean H score DOK1: 4.02, SD: 1.52; 2.33, SD: 2.11; $p=0.0044$ (Fig. 2, Table II).

A separate analysis of TA and SL revealed a lower expression of DOK1 and MTMR7 in the patients with multiple simultaneous serrated lesions ($n=35$) compared to patients with a single lesion ($n=58$) (Fig 3).

DOK1 showed a lower expression in both dysplastic (single lesion vs. multiple lesions; median H score: 3.82, SD: 1.85; 1.92, SD: 1.76; $p<0.0001$) and adjacent non-dysplastic tissue (single lesion vs. multiple lesions; median H score: 4.05, SD: 2.22, 2.33, SD: 2.31; $p=0.0099$) in patients with > 1 serrated lesion. Expression of MTMR7 was also significantly lower in dysplastic tissue of patients with simultaneous hyperplastic lesions (single lesion vs. multiple lesions median H score: 1.80, SD: 1.54; 0.92, SD: 1.95; $p=0.0207$), but not in surrounding non-dysplastic tissue. No difference of expression was observed in non-dysplastic tissue (Figs. 2 and 5).

DOK1 Expression in Serrated Lesions

A comparison between TA and SL showed a lower DOK1 expression in dysplastic tissue of SL (TA vs. SL; median H score TA: 3.67, SD: 1.98; 1.98, SD: 2.26; $p=0.0026$) (Fig. 3). For MTMR7, no differences were detected (Fig. 3). Logistic regression analysis revealed an increased likelihood of

Table II. Survey of protein expression of DOK1 & MTMR7 in adenoma/polyps (dysplasia) and adjacent non-dysplastic tissue and association with clinical variables

Characteristic		Marker							
		DOK1				MTMR7			
		Dysplasia	p	Non-dysplasia	p	Dysplasia	p	Non-dysplasia	p
Age	< 62 years (n=27)	2.29±2.23	0.0469*	3.17±2.44	0.5557	1.20±1.97	0.6671	2.00±2.35	0.0544
	> 62 years (n=29)	3.65±1.64		4.00±2.24		1.63±1.45		1.00±1.71	
Gender	Male (n=33)	3.17±1.74	0.3795	4.00±2.24	0.6781	1.75±1.90	0.0318*	1.17±2.20	0.4483
	Female (n=23)	3.80±2.31		3.00±2.49		1.20±1.18		1.00±1.85	
Size	> 20mm (n=32)	4.02±1.52	0.0044**	4.25±2.22	0.2355	1.70±1.46	0.4165	1.00±1.27	0.1516
	< 20mm (n=58)	2.33±2.11		3.67±2.41		1.23±1.80		1.25±2.31	
Histology	TA (n=48)	3.67±1.98	0.0026*	4.00±2.13	0.3852	1.33±1.42	0.9967	1.00±1.14	0.0837
	SL (n=42)	1.98±2.26		3.33±2.54		1.40±2.04		1.00±2.55	
Localisation	Left (n=47)	3.00±2.17	0.6255	3.00±2.28	0.0091 *	1.23±1.86	0.1442	1.00±2.30	0.0104*
	Right (n=43)	3.40±1.82		4.50±2.22		1.68±1.56		2.00±1.81	

Results are displayed as mean H scores ± standard deviation. Age of patients was categorized by the median age of 62 years. A diameter of 20 mm was applied to categorize adenomas into small (< 20mm) or big lesions (> 20mm). TA: Traditional adenoma; SL: Serrated lesion. *: $p<0.05$, **: $p<0.01$.

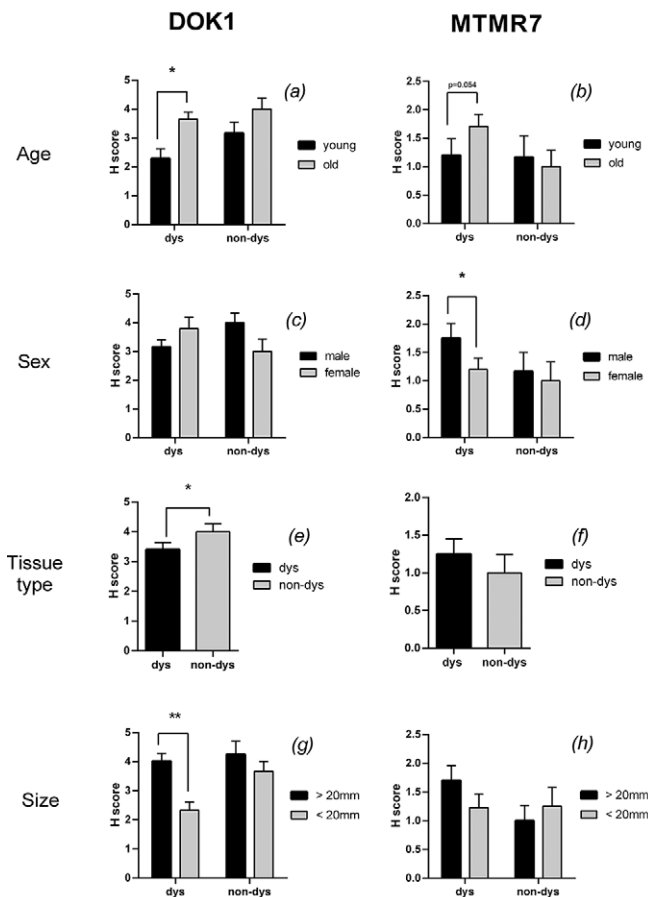


Fig. 2. Results for expression of DOK1 and MTMR7 in dysplastic tissue (dys) and adjacent non-dysplastic tissue (non-dys). Significance level 0.05, *: $p < 0.05$, **: $p < 0.01$. Results are displayed as median H score (Immunohistochemical staining score) $\pm$ SD. Categorized by the median age, adenomas of young patients (< 62 y, $n=27$) had lower DOK1 expression than old patients (> 62 y, $n=29$) (a), a similar trend was seen for MTMR7 (b). Male patients ($n=33$) showed higher expression of MTMR7 in dysplastic tissue than female patients ($n=23$) (d), no sex-related difference for DOK1 was observed (c). While no difference for MTMR7 between dysplastic tissue and adjacent non-dysplastic tissue was observed (f), DOK1 (e) showed significantly lower expression in adenomas/polyps. In a binary classification of big ($n=32$) and small ($n=58$) adenomas (diameter more or less than 20mm), DOK1 (g) showed higher expression in big adenomas than in small adenomas. MTMR7 showed no difference regarding size (h).

serrated histology in the case of high DOK1 expression in the dysplastic and adjacent non-dysplastic tissue on a high confidence level [area under curve (AUC): 0.77; odds ratio (OR) for dysplastic tissue: 0.43, 95% confidence interval (CI): 0.26-0.68, $p=0.0004$; OR for non-dysplastic tissue: 1.63, 95%CI: 1.11-2.41, $p=0.0129$] (Fig. 4). In addition, a Fisher's exact test confirmed the dependency of serrated histology on DOK1 expression ($p=0.05$).

DOK1 and MTMR7 Expression in Segmental Recurrence

A relapse of the adenoma in the same colon segment experienced 19 of 56 patients. Those had a higher level of DOK1 in dysplastic (relapse vs. no-relapse; median H score: 4.62, SD: 1.80; mean H score: 3.00, SD: 1.72, $p=0.0481$) and adjacent non-dysplastic tissue (relapse vs. no-relapse; median H score: 5.00, SD: 2.22, 3.00, SD: 2.13; $p=0.0291$). Difference

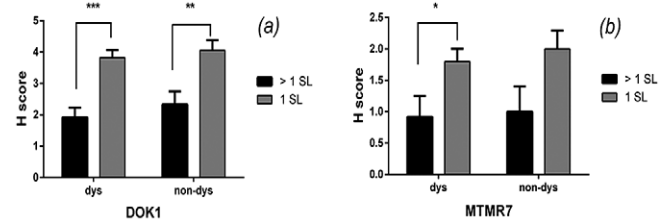


Fig. 3. Expression of DOK1/MTMR7 and simultaneous serrated lesions (SL). DOK1 was profoundly reduced both in dysplastic (dys) and adjacent non-dysplastic (non-dys) tissue in patients with more than 1 SL, ***, $p < 0.001$, **, $p < 0.01$ (a). MTMR7 was also reduced in dysplastic tissue (dys), but not in non-dysplastic tissue (non-dys), *, $p < 0.05$ (b). Results are displayed as mean H score (immunohistochemical staining score) $\pm$ SD.

in expression was even higher in non-dysplastic tissue in older patients as separated by mean age (segmental relapse vs. no segmental relapse; median H score: 5.50, SD: 1.99; 3.00, SD: 1.99, $p=0.0054$) (Fig. 5).

In contrast, MTMR7 showed a lower expression in the adjacent mucosa of patients with relapse in an adenoma-related analysis (relapse vs. no relapse; median H score: 1.00, SD 1.12; 1.83, SD: 2.40, $p=0.0362$) and a tendency to lower expression in dysplastic tissue (relapse vs. no relapse; median H score: 1.20, SD: 1.55; 1.88, SD: 1.80, $p=0.0597$) (Fig. 5). In dysplastic tissue, expression of MTMR7 decreased the probability of relapse in the same colon segment in an adenoma-related analysis (MTMR AUC in ROC analysis: 0.76; OR: 7 0.43, 95%CI: 0.22-0.84, $p=0.0129$) (Table III).

MTMR7 Expression in Distant Recurrence

Within this cohort, 21 out of 56 patients experienced relapse in distant colon segments, and the case-related analysis revealed a lower level of MTMR7 expression in adjacent non-dysplastic tissue in relapse (distant relapse vs. no relapse; median H score: 0.90, SD: 1.90; 1.88, SD: 2.20; $p=0.0500$). A tendency to lower expression in dysplastic tissue of patients with recurrence was seen as well (distant recurrence vs. no recurrence; median H score: 1.03, SD 1.41; 1.92, SD: 1.82, $p=0.0615$). Lower MTMR7 expression in the tissue of patients with recurrence was also observed in the adenoma-related analysis for adjacent non-dysplastic (distant relapse vs. no relapse; median H score: 0.86, SD: 1.92; 1.67, SD: 2.10, $p=0.0144$) and by trend in dysplastic tissue (distant relapse vs. no relapse; median H score: 1.20, SD: 1.59; 1.75, SD: 1.76, $p=0.0704$) (Fig. 5). Chi-squared test showed a dependency of distant relapse and low MTMR7 expression in adjacent non-dysplastic tissue ($p=0.0144$) (Table III).

Predictive Value of DOK1 and MTMR7 Combination for Segmental Recurrence

Finally, we tested whether the combination of markers reveals a better predictive value for the likelihood of adenoma recurrence. Therefore, we performed logistic regression analysis for different marker combinations.

A stepwise selection model of logistic regression yielded a two-marker combination of DOK1 and MTMR7 both in adjacent non-dysplastic tissue in case-related analysis as

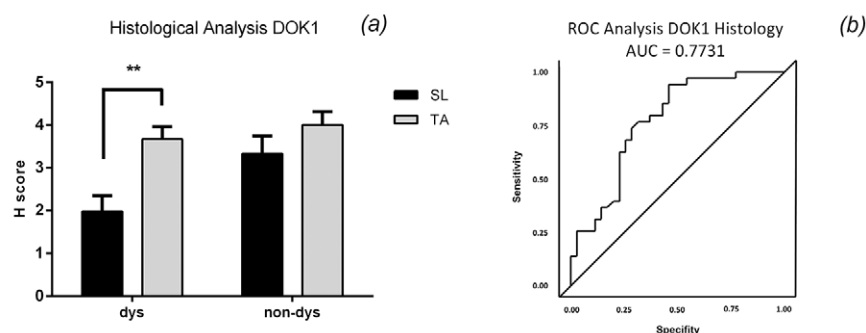


Fig. 4. DOK1 and serrated histology. Serrated lesions (SL, hyperplastic polyps and serrated adenoma, $n=42$) showed lower levels of DOK1 than traditional adenoma (tubular, villous, tubulo-villous, $n=48$). No difference was observed in adjacent non-dysplastic tissue (a), **: $p<0.01$. Odds ratio showed bigger likelihood of serrated histology upon high expression in adjacent non-dysplastic tissue (odds ratio non-dysplastic 1.63, CI 1.11-2.41, $p=0.0129$) with a good predictive value (ROC AUC 0.7731) (b). Results are displayed as mean H score (Immunohistochemical staining score) $\pm$ SD.

significant factors for adenoma recurrence in the same colon segment. Interestingly, expression of DOK1 increased, while MTMR7 decreased the probability of metachronous adenomas (AUC in ROC analysis: 0.78; DOK1 OR=1.62, 95%CI: 1.1-1.39, $p=0.0155$; MTMR7 OR=0.57, 95%CI: 0.37-1.00, $p=0.050$) (Fig. 5, Table IV).

DISCUSSION

We analysed the expression of DOK1 and MTMR7 in colorectal adenomas to find associations with clinical characteristics and histopathological subtypes. One major aim was to decipher the molecular patterns of recurrence. Hence, we performed immunohistochemical staining, digitally augmented microscopy for 96 colorectal polyps/adenomas as well as for adjacent mucosa and correlated the results with the clinical data.

In the last decades, there has been a rising incidence of early-onset CRC, which is defined by age at diagnosis < 50 years. Supposed risk factors are similar to late-onset CRC: lifestyle, western diet, alcohol and tobacco consumption [18]. On molecular levels, a multiomics approach linked inflammatory-driven oxidative stress to early-onset CRC [19]. We found a significantly lower expression of DOK1 in adenomas of younger patients. Besides its influence on growth receptor signalling, DOK1 also regulates immunity pathways [20]. Accordingly, a knockout of *Dok1* and *Dok2* in

a mouse model caused severe colitis [21], which might point to a possibly augmented inflammatory-driven accelerated development of colorectal lesions in patients with an early loss of DOK1. Most professional societies recommend screening endoscopy for patients > 50 years, but if patients are examined at a younger age, a look at molecular expression patterns in normal mucosa and adenomas could help to stratify patient's risk and future tailored prevention.

Our cohort was undersized for the analysis of male/female relapse or gender-separated differences in histopathological subgroups, but we found a lower MTMR7 expression in female patients. Male gender was associated with earlier development and progression of colorectal adenomas [22, 23]. So far, little is known about gender-dependent differences in expression or function of oncogenes or tumor suppressors in gastrointestinal cancers. At least, a preclinical model of intestinal tumorigenesis of APC-driven polyposis showed a reduced susceptibility for radiation-induced intestinal lesions in female mice [24]. Since epidemiological data repeatedly showed gender differences in progression of adenoma, it would be of interest to have a larger scale analysis of the oncogenes/tumor suppressors and gender-dependency in the adenoma-carcinoma progression, which could improve tailored prevention strategies.

Taken together, we found conflicting results for DOK1 and its supposed role as tumor suppressor. DOK1 showed promoting and suppressive features. In an overall analysis, DOK1 had lower expression levels in dysplastic tissue as

Table III. Survey of protein expression analysis of DOK1 & MTMR7 in local, segmental and distant recurrence

Marker		Recurrence								
		Local			Segmental			Distant		
		Yes n=9	No n=47	p	Yes n=19	No n=37	p	Yes n=21	No n=35	p
DOK1	Dysplasia	4.40±1.24	3.33±2.07	0.1784	4.62±1.80	3.00±1.72	0.0481 *	3.83±1.86	3.40±1.72	0.4475
	Non-dysplasia	4.50±1.63	4.00±2.40	0.5231	5.00±2.22	3.00±2.13	0.0291 *	4.00±1.99	3.83±2.30	0.6956
MTMR7	Dysplasia	1.30±1.95	1.60±1.79	0.9313	1.20±1.55	1.88±1.80	0.0597#	1.03±1.41	1.92±1.82	0.0615
	Non-dysplasia	1.25±2.21	1.00±2.20	0.9552	1.00±1.12	1.83±2.40	0.0362# *	0.90±1.90	1.88±2.20	0.0500 *

Each marker was determined in dysplastic tissue of adenomas/polyps and adjacent non-dysplastic tissue. Results are displayed as mean H scores $\pm$ standard deviation. Analysis was case-related if not labelled otherwise (#: adenoma-related analysis). P values were calculated using Mann-Whitney U test. *: $p < 0.05$.

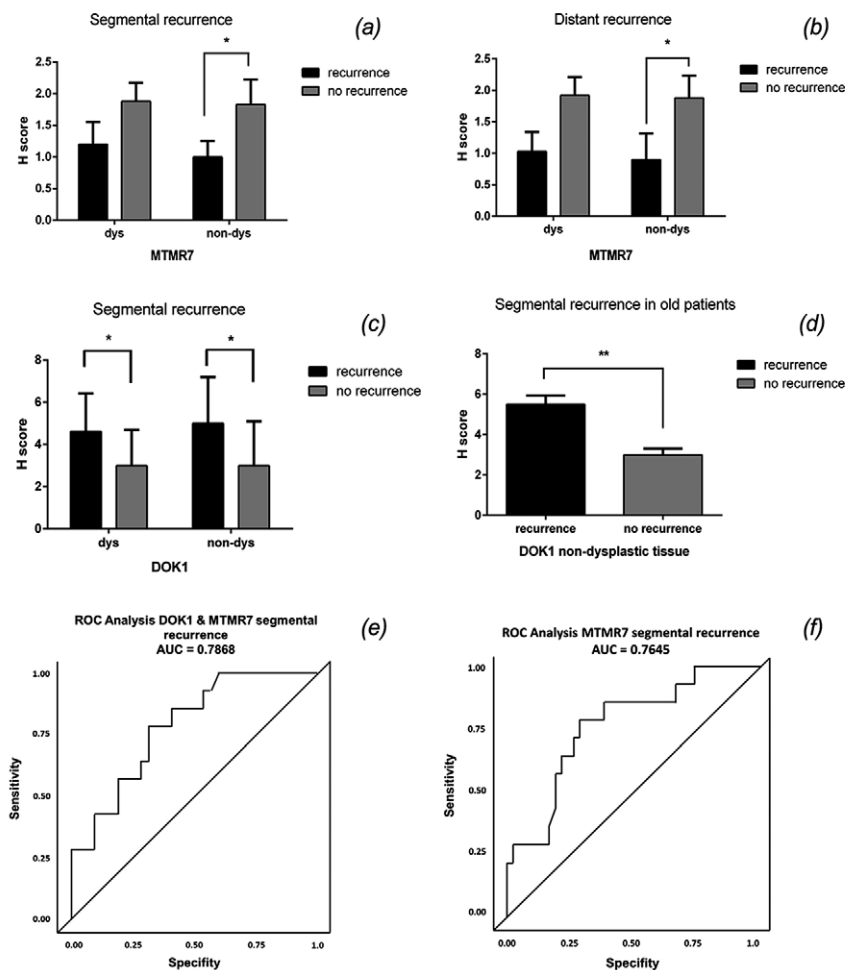


Fig. 5. Characteristics of DOK1/MTMR7 expression and recurrence. Patients with distant (a) and segmental recurrence (b) showed lower expression of MTMR7 in adjacent non-dysplastic tissue (non-dys) than patients without recurrence, *: $p < 0.05$ (a). No difference was observed in dysplastic tissue (dys). DOK1 was higher expressed both in dysplastic (dys) and adjacent non-dysplastic (non-dys) tissue (c). Difference in expression was even more significant in old patients (as subdivided by median age of 62y), **: $p < 0.01$ (d). In a-d, results are displayed as mean H score (Immunohistochemical staining score) $\pm$ SD. The combined expression profile of DOK1 and MTMR7 in adjacent non-dysplastic tissue showed a predictive value for segmental recurrence (e). ROC: receiver operator characteristics, AUC: area under the curve. In dysplastic tissue, a lower expression of MTMR7 increased was predictive for segmental recurrence (f).

compared with adjacent non-dysplastic tissue. DOK1 was higher expressed in big lesions, suggesting a role in tumor growth, but lower expressed in younger patients, suggesting an earlier onset when lost. Segmental recurrence was more likely with a higher expression of DOK1, again implicating a tumor promoting role. So far, there were only tumor-suppressive functions reported for DOK1 in CRC [14]. The heterogenous data presented here could be explained by time- and context-dependent changes in functions of DOK1, but determinants remain elusive. Context-dependency is a frequently observed phenomenon of cancer-related genes which include signalling networks and genetic interactions [25, 26]. Some of these context-dependencies could even be part of a selection process in tumorigenesis [27].

Our observations for MTMR7 seem to be more coherent to previous findings. It reduced the likelihood of segmental recurrence, and the distant recurrence was significantly

associated with lower MTMR7, a lipid phosphatase that can activate peroxisome proliferator-activated receptor gamma (PPAR γ) and inhibit proliferation in CRC models [15, 28]. The observation of lower MTMR7 in “normal” mucosa of patients with recurrence suggests that a loss of MTMR7 may be an early event in carcinogenesis.

Most of our patients did not have a history of inflammatory bowel disease, but there is growing evidence of inflammatory contribution to colorectal neoplasia in non-IBD patients [29].

Context-dependency of the immune system was observed in the case of DOK1 with pro- and anti-inflammatory features. While a recent study reported pro-inflammatory functions of DOK1 on macrophages in gastric cancer [30], others showed that DOK1 can negatively regulate immune receptor signalling in cancer [20]. These immune-related questions on the exact role of DOK1 in immune-related adenoma progression/recurrence cannot be further addressed with our data since we

Table IV. Logistic regression using a stepwise selection model for segmental recurrence

Stepwise selection model of logistic regression for segmental recurrence							
Case-related analysis of DOK1 & MTMR7							
	Tissue type	Odds ratio	95%CI	Coefficient	SE	p value	ROC AUC
DOK1	Non-dysplastic	1.62	1.1–1.39	0.482	0.199	0.0155 *	0.78
MTMR7	Non-dysplastic	0.57	0.95–1.98	-0.561	0.286		
Adenoma-related analysis of MTMR7							
	Tissue type	Odds ratio	95% CI	Coefficient	SE	p value	ROC AUC
MTMR7	Dysplastic	0.43	0.22 – 0.84	-0.846	0.340	0.0129 *	0.76

Confidence level 95%. CI: confidence interval, SE: standard error, ROC: receiver operator characteristics, AUC: area under the curve. * = $p < 0.05$.

lack information on the inflammatory status of our samples, but it would be of interest to correlate DOK1 expression in different stages of adenoma to the inflammatory features.

Another question to address: When do these tumor suppressors get lost in the adenoma-carcinoma sequence or in serrated pathway respectively? Is the different time point and state of progression the discriminator between high and low expression or pro- or anti-tumor effects? Answers could be found in longer lasting, slower growing tumor models or more frequent in-adenoma biopsies under close surveillance.

BRAF mutations are the main drivers of serrated histology, while *KRAS* mutations seem to be less important in this pathway [4]. DOK1 has shown its ability to inhibit the EGFR-RAS-ERK1/2 signalling cascade by different mechanisms, partly depending on its subcellular localization [14, 31]. Little is known about the influence of DOK1 on *BRAF*, but since *BRAF* is downstream of RAS, interactions on *BRAF* are conceivable. Our observation of lower DOK1 in SL than in TA, contingency of serrated histology and DOK1 expression, reduction of DOK1 in simultaneous SL and a high predictive value of expression of DOK1 for serrated histology supports the assumption of a causal interrelation.

An interesting finding is the promising predictive value for adenoma recurrence by the combination of two molecular markers. Surprisingly, DOK1 and MTMR7 showed an opposite pattern, but increased DOK1 and decreased MTMR7 in adjacent “normal” mucosa could robustly predict segmental relapse.

Histologically non-dysplastic mucosa, adjacent to adenomas or not, may already harbour genetic alterations which are not sufficient to influence morphology but likely influence transformation to adenomas [32]. This observation led to the concept of field cancerization, which is a controversy in science [33, 34]. However, the results of our study with lower levels of DOK1 in adjacent “normal” mucosa fits this concept, as our findings of a higher expression of DOK1 in non-dysplastic adjacent tissue of patients with segmental recurrence. In addition, we observed a lower expression of MTMR7 in non-dysplastic adjacent mucosa from patients who experienced a relapse in distant colon segments. Unfortunately, we did not have age-matched normal colon controls. The supplementary normal colon tissue arrays showed expression of both DOK1 and MTMR7 in normal mucosa and in normal mucosa adjacent to tumor (NAT). NAT sample size was too small to perform statistics and the age of NAT patients was higher than donors of normal tissue, but at least for MTMR7 there might be a tendency to decreased expression from normal

tissue to NAT to tumor tissue, an observation which should be validated in larger age- and case-matched cohorts.

Thus, expression of cancer-related genes in adjacent tissue maybe more crucial for metachronous lesions than in dysplastic tissue.

A recent study on surveillance colonoscopy revealed a high frequency of either too early follow-up examination or not conducted follow-up endoscopies [35], and overuse was common by diverse reasons [36, 37]. Conducting follow-up colonoscopies after an appropriate interval could be achieved by including more patient's characteristics such as age, gender and molecular patterns. Larger studies are required for identification of such patterns, but in the end, a personalized follow-up interval could improve recommendations, save resources and sustain a patient's acceptance.

CONCLUSIONS

This work is an observational study with limited causal implications. Nevertheless, we demonstrate another example of context and time dependency of supposed anti-tumoral factors, we shed light on the relevance of DOK1 in the serrated pathway to CRC and highlight the role of the “normal mucosa” in adenoma development. This work exemplarily shows the potential of molecular markers to predict recurrence of colorectal adenoma. This might open the field for novel markers of recurrence and tailored prevention strategies for adjusted and better surveillance.

Conflicts of interest: None to declare.

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REFERENCES

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018;68:394-424. doi:[10.3322/caac.21492](https://doi.org/10.3322/caac.21492)
- Zhan T, Hahn F, Hielscher T, et al. Multiple behavioral factors are associated with occurrence of large, flat colorectal polyps. *Int J Colorectal Dis* 2017;32:575-582. doi:[10.1007/s00384-016-2717-0](https://doi.org/10.1007/s00384-016-2717-0)
- Lin SH, Raju GS, Huff C, et al. The somatic mutation landscape of premalignant colorectal adenoma. *Gut* 2018;67:1299-1305. doi:[10.1136/gutjnl-2016-313573](https://doi.org/10.1136/gutjnl-2016-313573)
- Kim SY, Kim TI. Serrated neoplasia pathway as an alternative route of colorectal cancer carcinogenesis. *Intest Res* 2018;16:358-365. doi:[10.5217/ir.2018.16.3.358](https://doi.org/10.5217/ir.2018.16.3.358)
- Nishihara R, Wu K, Lochhead P, et al. Long-term colorectal-cancer incidence and mortality after lower endoscopy. *N Engl J Med* 2013;369:1095-1105. doi:[10.1056/NEJMoa1301969](https://doi.org/10.1056/NEJMoa1301969)
- Zauber AG, Winawer SJ, O'Brien MJ, et al. Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. *N Engl J Med* 2012;366:687-696. doi:[10.1056/NEJMoa1100370](https://doi.org/10.1056/NEJMoa1100370)
- Zhan T, Hielscher T, Hahn F, et al. Risk Factors for Local Recurrence of Large, Flat Colorectal Polyps after Endoscopic Mucosal Resection. *Digestion* 2016;93:311-317. doi:[10.1159/000446364](https://doi.org/10.1159/000446364)
- Belderbos TDG, Leenders M, Moons LMG, Siersema PD. Local recurrence after endoscopic mucosal resection of nonpedunculated colorectal lesions: systematic review and meta-analysis. *Endoscopy* 2014;46:388-402. doi:[10.1055/s-0034-1364970](https://doi.org/10.1055/s-0034-1364970)
- Fiedler D, Heselmeyer-Haddad K, Hirsch D, et al. Single-cell genetic analysis of clonal dynamics in colorectal adenomas indicates CDX2 gain as a predictor of recurrence. *Int J Cancer* 2019;144:1561-1573. doi:[10.1002/ijc.31869](https://doi.org/10.1002/ijc.31869)
- Lee S, Huang H, Niu Y, Tommasino M, Lenoir G, Sylla BS. Dok1 expression and mutation in Burkitt's lymphoma cell lines. *Cancer Lett* 2007;245:44-50. doi:[10.1016/j.canlet.2005.10.045](https://doi.org/10.1016/j.canlet.2005.10.045)
- Berger AH, Niki M, Morotti A, et al. Identification of DOK genes as lung tumor suppressors. *Nat Genet* 2010;42:216-223. doi:[10.1038/ng.527](https://doi.org/10.1038/ng.527)
- Shinohara H, Yasuda T, Yamanashi Y. Dok-1 tyrosine residues at 336 and 340 are essential for the negative regulation of Ras-Erk signalling, but dispensable for rasGAP-binding. *Genes Cells* 2004;9:601-607. doi:[10.1111/j.1356-9597.2004.00748.x](https://doi.org/10.1111/j.1356-9597.2004.00748.x)
- Songyang Z, Yamanashi Y, Liu D, Baltimore D. Domain-dependent function of the rasGAP-binding protein p62Dok in cell signaling. *J Biol Chem* 2001;276:2459-2465. doi:[10.1074/jbc.M005504200](https://doi.org/10.1074/jbc.M005504200)
- Friedrich T, Söhn M, Gutting T, et al. Subcellular compartmentalization of docking protein-1 contributes to progression in colorectal cancer. *EBioMedicine* 2016;8:159-172. doi:[10.1016/j.ebiom.2016.05.003](https://doi.org/10.1016/j.ebiom.2016.05.003)
- Weidner P, Söhn M, Gutting T, et al. Myotubularin-related protein 7 inhibits insulin signaling in colorectal cancer. *Oncotarget* 2016;7:50490-50506. doi:[10.18632/oncotarget.10466](https://doi.org/10.18632/oncotarget.10466)
- Schmiegel W, Buchberger B, Follmann M, et al. S3-Leitlinie – Kolorektales Karzinom. *Z Gastroenterol* 2017;55:1344-1498. doi:[10.1055/s-0043-121106](https://doi.org/10.1055/s-0043-121106)
- McCarty KS Jr, Miller LS, Cox EB, Konrath J, McCarty KS Sr. Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. *Arch Pathol Lab Med* 1985;109:716-721.
- Burnett-Hartman AN, Lee JK, Demb J, Gupta S. An update on the epidemiology, molecular characterization, diagnosis, and screening strategies for early-onset colorectal cancer. *Gastroenterology* 2021;160:1041-1049. doi:[10.1053/j.gastro.2020.12.068](https://doi.org/10.1053/j.gastro.2020.12.068)
- Holowatyj AN, Gigic B, Herpel E, et al; MetaboCCC Consortium; ColoCare Study. Distinct Molecular Phenotype of Sporadic Colorectal Cancers Among Young Patients Based on Multiomics Analysis. *Gastroenterology* 2020;158:1155-1158.e2. doi:[10.1053/j.gastro.2019.11.012](https://doi.org/10.1053/j.gastro.2019.11.012)
- Mashima R, Hishida Y, Tezuka T, Yamanashi Y. The roles of Dok family adapters in immunoreceptor signaling. *Immunol Rev* 2009;232:273-285. doi:[10.1111/j.1600-065X.2009.00844.x](https://doi.org/10.1111/j.1600-065X.2009.00844.x)
- Waseda M, Arimura S, Shimura E, Nakae S, Yamanashi Y. Loss of Dok-1 and Dok-2 in mice causes severe experimental colitis accompanied by reduced expression of IL-17A and IL-22. *Biochem Biophys Res Commun* 2016;478:135-142. doi:[10.1016/j.bbrc.2016.07.079](https://doi.org/10.1016/j.bbrc.2016.07.079)
- Krigel A, Zhou M, Terry MB, Kastrinos F, Lebwohl B. Symptoms and demographic factors associated with early-onset colorectal neoplasia among individuals undergoing diagnostic colonoscopy. *Eur J Gastroenterol Hepatol* 2020;32:821-826. doi:[10.1097/meg.0000000000001720](https://doi.org/10.1097/meg.0000000000001720)
- Adán Merino L, Mercedes AM, Jose BA, Ana BM, Sonia MC. Factors related to colorectal cancer in advanced adenomas and serrated polyps: a further step toward individualized surveillance. *Eur J Gastroenterol Hepatol* 2018;30:1337-1343. doi:[10.1097/meg.0000000000001227](https://doi.org/10.1097/meg.0000000000001227)
- Trani D, Moon BH, Kallakury B, Hartmann DP, Datta K, Fornace AJ Jr. Sex-dependent differences in intestinal tumorigenesis induced in Apc1638N/+ mice by exposure to γ rays. *Int J Radiat Oncol Biol Phys* 2013;85:223-229. doi:[10.1016/j.ijrobp.2012.02.053](https://doi.org/10.1016/j.ijrobp.2012.02.053)
- Henkel L, Rauscher B, Boutros M. Context-dependent genetic interactions in cancer. *Curr Opin Genet Dev* 2019;54:73-82. doi:[10.1016/j.gde.2019.03.004](https://doi.org/10.1016/j.gde.2019.03.004)
- Sharma S, Petsalaki E. Large-scale datasets uncovering cell signalling networks in cancer: context matters. *Curr Opin Genet Dev* 2019;54:118-124. doi:[10.1016/j.gde.2019.05.001](https://doi.org/10.1016/j.gde.2019.05.001)
- Vibishan B, Watve MG. Context-dependent selection as the keystone in the somatic evolution of cancer. *Sci Rep* 2020;10:4223. doi:[10.1038/s41598-020-61046-7](https://doi.org/10.1038/s41598-020-61046-7)
- Weidner P, Söhn M, Schroeder T, et al. Myotubularin-related protein 7 activates peroxisome proliferator-activated receptor-gamma. *Oncogenesis* 2020;9:59. doi:[10.1038/s41389-020-0238-8](https://doi.org/10.1038/s41389-020-0238-8)
- Shi Y, Li Z, Zheng W, et al. Changes of immunocytic phenotypes and functions from human colorectal adenomatous stage to cancerous stage: Update. *Immunobiology* 2015;220:1186-1196. doi:[10.1016/j.imbio.2015.06.003](https://doi.org/10.1016/j.imbio.2015.06.003)
- Li T, Li B, Sara A, et al. Docking protein-1 promotes inflammatory macrophage signaling in gastric cancer. *Oncoimmunology* 2019;8:e1649961. doi:[10.1080/2162402x.2019.1649961](https://doi.org/10.1080/2162402x.2019.1649961)
- Burgermeister E, Friedrich T, Hitkova I, et al. The Ras inhibitors caveolin-1 and docking protein 1 activate peroxisome proliferator-activated receptor γ through spatial relocation at helix 7 of its ligand-binding domain. *Mol Cell Biol* 2011;31:3497-3510. doi:[10.1128/mcb.01421-10](https://doi.org/10.1128/mcb.01421-10)
- Patel A, Tripathi G, Gopalakrishnan K, Williams N, Arasaradnam RP. Field cancerisation in colorectal cancer: a new frontier or pastures past? *World J Gastroenterol* 2015;21:3763-3772. doi:[10.3748/wjg.v21.i13.3763](https://doi.org/10.3748/wjg.v21.i13.3763)
- Rosa I, Fidalgo P, Chaves P, Pereira AD. The co-localization of carcinomas and adenomas favors a regional field defect in the colon: an observational study. *Int J Colorectal Dis* 2015;30:323-327. doi:[10.1007/s00384-014-2087-4](https://doi.org/10.1007/s00384-014-2087-4)

34. Zauber P, Marotta S, Sabbath-Solitare M. Molecular genetic changes in benign colorectal tumors synchronous with microsatellite unstable carcinomas do not support a field defect. *Int J Mol Epidemiol Genet* 2017;8:27-39.
35. Murphy CC, Sandler RS, Grubber JM, Johnson MR, Fisher DA. Underuse and Overuse of Colonoscopy for Repeat Screening and Surveillance in the Veterans Health Administration. *Clin Gastroenterol Hepatol* 2016;14:436-444.e1. doi:[10.1016/j.cgh.2015.10.008](https://doi.org/10.1016/j.cgh.2015.10.008)
36. Kruse GR, Khan SM, Zaslavsky AM, Ayanian JZ, Sequist TD. Overuse of colonoscopy for colorectal cancer screening and surveillance. *J Gen Intern Med* 2015;30:277-283. doi:[10.1007/s11606-014-3015-6](https://doi.org/10.1007/s11606-014-3015-6)
37. Anderson JC, Baron JA, Ahnen DJ, et al. Factors Associated With Shorter Colonoscopy Surveillance Intervals for Patients With Low-Risk Colorectal Adenomas and Effects on Outcome. *Gastroenterology* 2017;152:1933-1943.e5. doi:[10.1053/j.gastro.2017.02.010](https://doi.org/10.1053/j.gastro.2017.02.010)