

# Dickkopf-3 as a new potential marker for neoangiogenesis in colorectal cancer: Expression in cancer tissue and adjacent non-cancerous tissue<sup>1</sup>

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**Abstract.** Gene expression of Dickkopf-3 (Dkk-3) has been shown to be upregulated in tumor endothelium of colorectal cancer (CRC). For the first time, we analyzed Dkk-3 protein expression in CRC and its potential as a marker for neoangiogenesis. We used tissue microarrays (TMAs) to investigate Dkk-3 in microvessels of 403 CRC samples, 318 appropriate adjacent non-cancerous samples and 127 normal colorectal samples. Of cancer samples with CD31-positive microvessels, 67.7% were positive for Dkk-3. Dkk-3 staining was demonstrated in endothelial cells of *all* microvessels in nearly all cases. Dkk-3-positive samples showed a higher mean microvessel count than did Dkk-3-negative samples ( $P = 0.001$ ). Dkk-3 expression increased with rising numbers of microvessels per sample ( $P < 0.0001$ ). In adjacent samples with CD31-positive microvessels, 56% were Dkk-3-positive in *all* microvessels. Similar to cancer samples, Dkk-3-positive adjacent samples had a higher mean microvessel count than did Dkk-3-negative samples ( $P < 0.0001$ ), and Dkk-3 expression also increased with rising numbers of microvessels ( $P < 0.0001$ ). All microvessels in normal mucosa samples were negative for Dkk-3.

Dkk-3 can be considered a putative pro-angiogenic protein in neovascularization and may possibly be a marker for neoangiogenesis in CRC. Further investigations will elucidate whether Dkk-3 is a target structure for novel therapies.

Keywords: Dickkopf-3, neoangiogenesis, colorectal cancer, adjacent tissue, tissue microarrays

## 1. Introduction

Advances in multidisciplinary treatment and intensive follow-up programs have improved the outcome

of colorectal cancer patients [1–3]. However, tumor invasion, lymphatic spread and distant metastasis still limit the prognosis of colorectal cancer. Neoangiogenesis is a hallmark of most malignancies, and inhibition of this process is considered a promising strategy for cancer treatment. Folkman first mentioned that tumor progression is dependent on the development of new blood vessels [4]. Environmental and genetic alterations induce an “angiogenic switch” during tumor growth, with either upregulation of angiogenic or

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downregulation of antiangiogenic factors [5,6]. Several differences between tumor-associated endothelium in abnormal microvessels and normal endothelium have been established by studies of their structure and function [7]. St. Croix et al. revealed 46 genes showing stronger expression in tumor endothelium of colorectal cancer tissues than in endothelium of normal colonic mucosa. These genes, termed tumor endothelial markers (TEMs), show that tumor-associated and normal endothelium differ at the molecular level [8].

Dickkopf-3 (Dkk-3) – one of these TEMs – has been isolated according to its sequence homology to the cysteine-rich Dickkopf family of secreted canonical Wnt-inhibitors. The human Dickkopf (hDkk) family includes Dkk-1, Dkk-2, Dkk-3, Dkk-4 and a unique Dkk-3-related protein called Soggy (Sgy) [9]. Human Dkks 1–4 are glycoproteins of 255–350 amino acids that contain a signal sequence and share two conserved cysteine-rich domains, each of which displays a characteristic spacing of cysteines and other conserved amino acids. The N-terminal cysteine-rich domain – Dkk<sub>N</sub> (formerly termed Cys1) – is unique to the Dkks, while the C-terminal cysteine-rich domain – formerly termed Cys2 – has a pattern of ten cysteines related to that of the colipase family. These two domains are separated by a non-conserved linker region [10]. Dkk-3 is the most divergent of the four human Dkks and possesses an extended N-terminal domain, that precedes the Cys-1 domain, and an extended C-terminal region, that follows Cys-2 [9]. Dkk-1 and Dkk-4 have been found to inhibit Wnt signaling by binding to the transmembrane receptors Kremen 1 and 2 (Krm1/2) and lipoprotein receptor-related protein 5/6 (LRP5/6), a coreceptor of the Wnt/Fz receptor [10,11]. In contrast, the biological function and interaction partners of Dkk-3 remain unclear. There is no evidence that Dkk-3 is involved in inhibition of the WNT signaling pathway and thus the beta-catenin destabilization process [11]. However, gene expression of Dkk-3 has been shown to be upregulated in tumor endothelium of colorectal cancer tissue [8].

To our best knowledge, Dkk-3 protein expression in tumor microvessels has not been investigated in colorectal cancer so far. Thus, the current study was designed to analyze the role of Dkk-3 as a potential factor in neoangiogenesis in colorectal cancer. For a high-throughput approach we employed tissue microarrays (TMAs) containing a large series of colorectal cancer tissue and adjacent non-cancerous tissue.

## 2. Materials and methods

### 2.1. Specimens and tissue microarray (TMA) construction

Colorectal cancer specimens ( $n = 403$ ) and corresponding colorectal adjacent non-cancerous tissue specimens ( $n = 318$ ) from patients who underwent oncologic resection of colorectal cancer at Innsbruck Medical University were utilized for the study. The 403 patients were treated between 1992 and 2001 [224 men (55.6%) and 179 women (44.4%) at a mean age of 69.1 years (95% CI: 67.9–70.2)]. Resected tumors were histopathologically examined according to the guidelines of the Union Internationale Contre le Cancer (UICC) and were graded according to WHO classification. Chemotherapy was recommended to patients with UICC stage III/IV. Demographic data of all patients were recruited from our computerized surgical documentation system for colorectal cancer (ChiBASE). All patients were subsequently followed up as described elsewhere [3].

Formalin-fixed and paraffin-embedded specimens and corresponding hematoxylin-erythrosin (HE) staining sections were obtained from the Department of Pathology. Firstly, HE staining sections were observed and representative areas of cancer and adjacent non-cancerous tissue marked. Then tissue cylinders (0.6 mm in diameter) were punched from the corresponding region of paraffin samples using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD). The empty TMA paraffin block (25 mm × 35 mm) was holed through Tissue Arrayer (0.6 mm in diameter), and the tissue cylinders were neatly inserted in these holes. A different kind of tissue was also inserted as a marker, namely spleen tissue in this study. Each TMA paraffin block was sliced into 3- $\mu$ m-thick sections that were transferred to glass slides using the Paraffin Sectioning Aid System (Instrumedics, Hackensack, NJ).

In addition, a tissue microarray (Normal colon/rectum tissue multi-sites tissue microarray; diameter: 1.5 mm; thickness: 5  $\mu$ m) with normal colorectal mucosal specimens ( $n = 127$ ; male/female:  $n = 82/45$ ; colon/rectum:  $n = 58/69$ ) were purchased from US Biomax Inc. (Rockville, MD, USA).

### 2.2. Immunohistochemistry

TMA paraffin sections (cancer tissue sections, adjacent non-cancerous tissue sections and normal col-

orectal mucosa sections) were deparaffinized and rehydrated with xylene and graded alcohol series. Thereafter, antigens were retrieved by microwave treatment in citrate-buffer (10 mM, pH 6.0) and endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub>/methanol. Sections were incubated for 45 min in blocking solution containing 10% bovine calf serum (Dako Cytomation, Copenhagen, Denmark) and then stained for 1h with a primary polyclonal antiserum directed against human Dkk-3 (goat-anti-human Dkk-3 polyclonal 1.5 µg/mL; R&D Systems Inc., Minneapolis, USA). Primary antiserum was detected by incubation with a biotinylated secondary antibody (biotinylated horse-anti-goat IgG, Vector Laboratories Inc., Burlingame, CA, USA) with the Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) and the FAST DAB Tablet Set (Sigma, Vienna, Austria). The sections were washed with water, counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and mounted with medite Pertex® (Medite Medizintechnik, Burgdorf, Germany). Specificity of antiserum against human Dkk-3 was proven by blocking experiments with an excess of recombinant human Dkk-3. Simultaneously, a second TMA section from each specimen was stained with CD31 to identify microvessels by immunostaining endothelial cells for CD31 antigen. CD31 was detected with a monoclonal mouse anti-human CD31 antibody diluted 1:40 (clone JC70A, Dako Cytomation, Copenhagen, Denmark) and the respective secondary antibody (biotinylated goat anti-mouse IgG, Vector Laboratories Inc., Burlingame, CA, USA).

### 2.3. Quantification of neoangiogenesis

All CD31-positive stained microvessels from cancer tissue and adjacent non-cancerous tissue samples were manually counted by two independent investigators (P.M. and A.A.); the whole sample was counted at x400 magnification. Any stained endothelial cell or cell-cluster was identified as an independent vessel, if these cells or cell-clusters were clearly separate from each other. The average count of both investigators was defined as "microvessel count per sample". Additionally, samples were grouped according to their microvessel count: < 10 microvessels per sample, ≥ 10 < 20 microvessels per sample and ≥ 20 microvessels per sample.

### 2.4. Evaluation of Dkk-3 expression

Two independent pathologists (P.M. and S.S.) with no prior knowledge of the clinical data analyzed TMA sections in parallel.

A sample was considered as Dkk-3 positive if more than 90% of the identified microvessels were stained, irrespective of staining intensity. A sample was considered as Dkk-3 negative if less than 10% of the identified microvessels were stained. If a sample showed Dkk-3 staining in 10% to 90% of the identified microvessels it was defined as intermediate staining.

### 2.5. Statistical analysis

Statistical analysis was performed with SPSS 14.0 (SPSS, Inc., Chicago, IL). Descriptive statistics for continuous measures (microvessel count) are given as the mean with the respective 95% confidence interval (CI) in parenthesis. For discrete data (Dkk-3 expression) frequency counts and percentages were tabulated. Results of the various estimates were compared using either the chi-squared test or One-way ANOVA. All data on the date and cause of death were confirmed by the "Tumorregister Tirol", a register kept by the Tyrolean government. Cumulative patient survival was estimated using the Kaplan-Meier method; for comparison of survival curves the log-rank test (Mantel-Haenszel method) was used. The Cox proportional hazards linear regression model was computed to determine in a forward stepwise procedure which factors were associated simultaneously with survival. Results were considered significant when  $P < 0.05$ .

## 3. Results

### 3.1. Expression of Dkk-3 protein in colorectal cancer tissue

None of the cancer samples showed Dkk-3 staining in colorectal carcinoma cells, either in cellular membrane, cytoplasm or in the nucleus (Fig. 1B).

Out of all cancer samples ( $n = 403$ ), 63.8% (257/403) presented with CD31-positive microvessels (Fig. 1A), 36.2% (146/403) had no microvessels. With regard to samples with microvessels, the mean microvessel count per sample was 5.59 vessels (95% CI: 4.92–6.26). Three groups were formed according to microvessel count (see "Materials and Methods"): less than 10 microvessels per sample were observed in

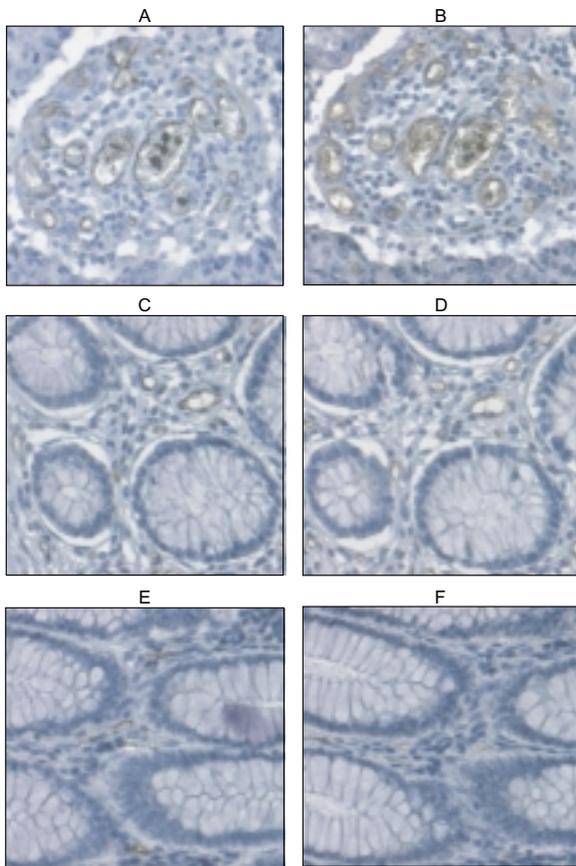


Fig. 1. CD31 and Dkk-3 expression in colorectal cancer tissue (A, B), colorectal adjacent non-cancerous tissue (C, D), and normal colorectal mucosa (E, F). A (CD31), B (Dkk-3): colorectal cancer tissue; C (CD31), D (Dkk-3): colorectal adjacent non-cancerous tissue; E (CD31), F (Dkk-3): normal colorectal mucosa.

73.5% (189/257),  $\geq 10$  but  $< 20$  microvessels in 17.5% (45/257) and  $\geq 20$  microvessels in 9% (23/257).

A positive staining reaction for Dkk-3 (Fig. 2) was found in 67.7% of samples with CD31-positive microvessels (174/257). No sample showed intermediate staining; and 32.3% (83/257) were Dkk-3 negative (Fig. 2). In terms of 174 Dkk-3 positive samples, 170 (97.7%) showed Dkk-3 expression in 100% of microvessels (Fig. 1B), four (2.3%) samples revealed Dkk-3 expression in more than 90% of microvessels. In all Dkk-3 negative samples 0% of identified microvessels were stained.

Furthermore, Dkk-3-positive samples showed a statistically significantly higher mean microvessel count [9.70 vessels (95% CI: 8.52–10.87)] than did Dkk-3-negative samples [6.82 vessels (95% CI: 6.34–7.30);  $F = 10.7$ ,  $DF = 1$ ,  $P = 0.001$ ]. Moreover, Dkk-3 expression within the three microvessel count groups

revealed a statistically significant increase with rising numbers of intratumoral microvessels per sample ( $\chi^2 = 450.1$ ,  $DF = 6$ ,  $P < 0.0001$ ; Fig. 3A).

Univariate analysis of clinicopathologic variables with regard to Dkk-3 expression is shown in Table 1. Statistically significant differences were observed for tumor site ( $\chi^2 = 7.9$ ,  $DF = 2$ ,  $P = 0.02$ ) and patient age ( $F = 4.1$ ,  $DF = 2$ ,  $P = 0.018$ ). A borderline correlation was found with pT stage ( $P = 0.054$ ), tumor differentiation ( $P = 0.079$ ) and tumor progression ( $P = 0.089$ ), respectively.

Survival analysis according to the Kaplan-Meier method showed a trend toward improved disease-free survival for patients with Dkk-3-negative samples (log-rank: 5.7,  $DF = 2$ ,  $P = 0.057$ ). Within this group, the five-year disease-free survival rate was 70% as compared with 59% for patients with Dkk-3-positive samples. Univariate survival analysis revealed T-stage, nodal status, metastases and tumor differentiation as significant predictors of disease-free survival (Table 2). Regarding overall survival, patients with Dkk-3-positive samples and Dkk-3-negative samples were comparable ( $p = 0.175$ ). Multivariate disease-free survival analysis showed nodal status and metastases as independent prognostic parameters (Table 3).

### 3.2. Expression of Dkk-3 protein in colorectal adjacent non-cancerous tissue

None of the adjacent non-cancerous tissue samples showed Dkk-3 staining in the glandular epithelium, either in cellular membrane, cytoplasm or in the nucleus (Fig. 1D).

CD31-positive microvessels were found in 84.3% (268/318) of adjacent non-cancerous tissue samples (Fig. 1C). The mean microvessel count per sample was 9.33 vessels (95% CI: 8.36–10.30). Again, three groups were formed according to microvessel count: less than 10 microvessels per sample were identified in 65.5% (176/268),  $\geq 10$  but  $< 20$  microvessels in 18% (48/268) and  $\geq 20$  microvessels in 16.5% (45/268). Fifty samples (15.7%) contained no microvessels.

In terms of CD31-positive samples, 56% (150/268; Fig. 2) showed a positive staining reaction for Dkk-3 in all microvessels (Fig. 1D), and 44% (118/268; Fig. 2) were negative for Dkk-3 in all microvessels. No sample showed intermediate staining.

Dkk-3-positive samples showed a statistically significantly higher mean microvessel count [14.51 (95% CI: 12.88–16.14)] than did Dkk-3-negative samples [6.64 (95% CI: 6.40–6.89);  $F = 93.2$ ,  $DF = 2$ ,  $P <$

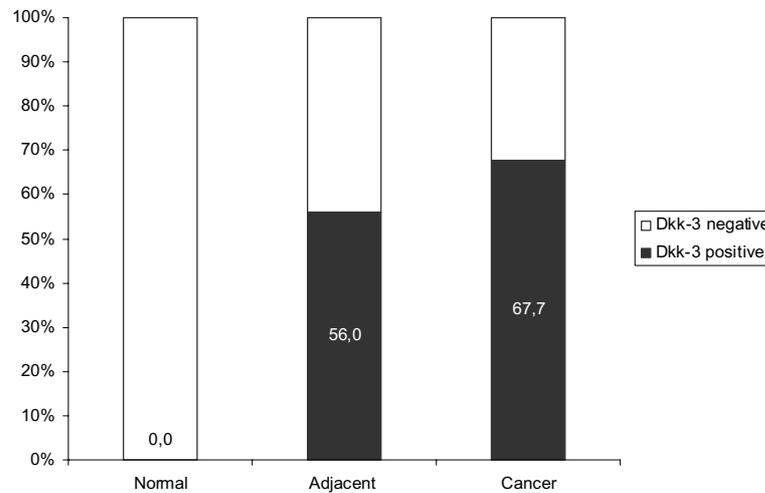


Fig. 2. Proportion of Dkk-3-positive and Dkk-3-negative samples in normal colorectal mucosa, colorectal adjacent non-cancerous tissue and colorectal cancer tissue.

0.0001]. In addition, Dkk-3 expression in the three microvessel count groups revealed a statistically significant increase with rising numbers of microvessels per sample ( $\chi^2 = 430.7$ ,  $DF = 6$ ,  $P < 0.0001$ ; Fig. 3B).

There were no significant correlations between Dkk-3 expression in adjacent non-cancerous tissue and the clinicopathologic variables described above.

### 3.3. Expression of Dkk-3 protein in normal colorectal mucosa

None of the 127 normal colorectal mucosal tissues showed a positive staining reaction for Dkk-3 protein, either in the CD31-positive endothelial cells of the microvessels or in the epithelial cells of the mucosa (Fig. 1. E, F and Fig. 2).

## 4. Discussion

In contrast to Dkk-1, Dkk-2, and Dkk-4, which have the ability to modulate the WNT/beta-catenin signaling pathway [10], the biological role of Dkk-3 remains unclear. However, Dkk-3 has been proposed to act as a suppressor of human tumor growth because it is downregulated in various types of cancer or tumor cell lines and its overexpression is associated with inhibition of cell proliferation [12–20]. With regard to colorectal cancer or colorectal tumor cell lines, no data are available on whether Dkk-3 is downregulated or upregulated. Byun et al. reported that there are no differences between Dkk-3 expression in colorectal cancer and in

matched normal colon tissue. In that study Dkk-3 was detected by in situ RNA hybridization in five of 14 normal colon tissues and four of 15 malignant colon tissue samples [21]. In the present study, which is the first analysis of Dkk-3 at the protein level in colorectal cancer, none of the 403 tumor samples showed Dkk-3 staining in colorectal carcinoma cells, either in cellular membrane, cytoplasm or in the nucleus. Furthermore, there was no positive staining reaction for Dkk-3 protein in the glandular epithelium of 127 normal colorectal mucosa samples. In comparison to other malignancies, Dkk-3 can be assumed to play a different role in colorectal tumor growth. For this reason, our study was designed to investigate the relationship between Dkk-3 and neoangiogenesis in colorectal cancer.

Angiogenesis is the driving force behind tumor growth and therefore one of the strongest features of prognostic relevance in cancer identified so far. Although angiogenesis initially develops by incorporating host blood vessels, solid tumors apparently cannot grow more than 1.0 mm<sup>3</sup> unless the tumor synthesizes its own new networks of morphologically and functionally abnormal microvessels [22,23]. This process of neovascularization requires a direct or indirect role of angiogenic factors [24,25] that are derived mainly from cancer cells and stimulate pre-existing host capillaries to induce endothelial cell proliferation, migration and, eventually, maturation into tube-like structures [26]. St. Croix et al. compared gene expression patterns of endothelial cells from blood vessels of normal and malignant colorectal tissues and revealed 46 tumor endothelial markers (TEMs) that were expressed at sub-

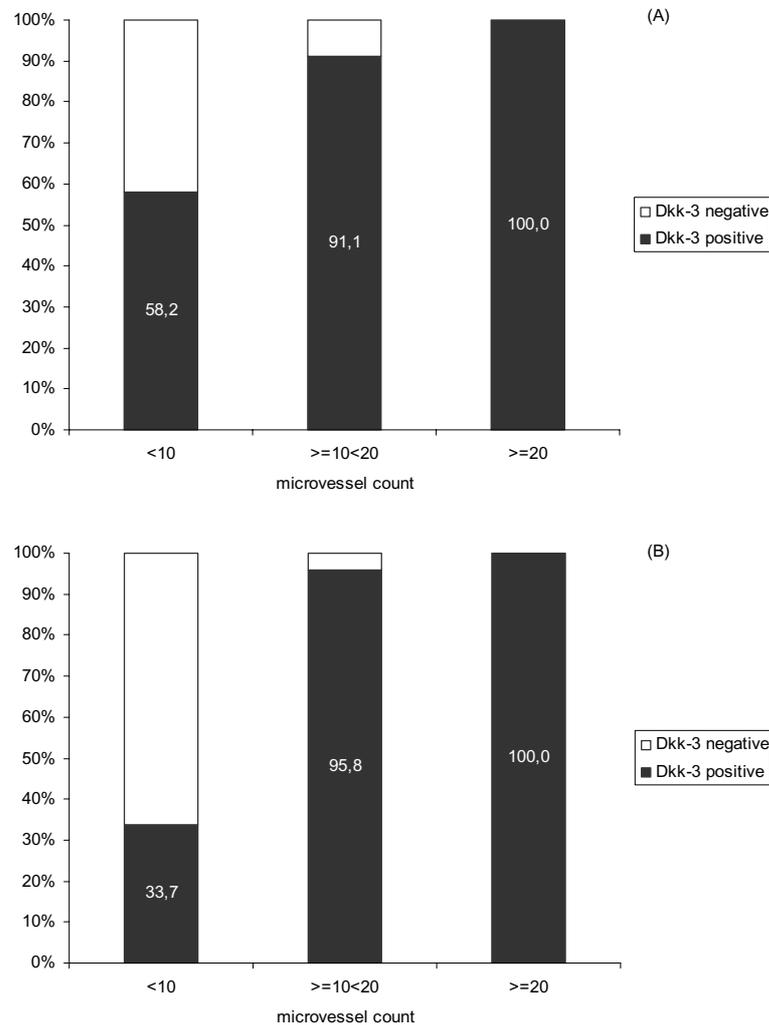


Fig. 3. Dkk-3 expression in colorectal cancer tissue 3A;  $P < 0.0001$ ) and colorectal adjacent non-cancerous tissue 3B;  $P < 0.0001$ ) in the three microvessel count groups.

stantially higher levels ( $> 10$ -fold) in tumor-associated endothelium [8]. Although that study showed that gene expression of Dkk-3 – as one of these TEMs - is strongly upregulated in colorectal cancer endothelium, further analyses were not performed. To our knowledge, the present study is the first investigation of Dkk-3 protein expression in tumor endothelium of colorectal cancer tissue versus colorectal adjacent non-cancerous tissue and normal colorectal mucosa. This study demonstrates that 174 (67.7%) out of 257 colorectal cancer samples with CD31-positive microvessels had a positive staining reaction for Dkk-3 in endothelial cells of all microvessels, whereas in samples of normal colorectal mucosa all CD31-positive microvessels lacked Dkk-3 staining. According to St. Croix's Science study

indicating that tumor and normal endothelium are distinct at the molecular level [8], our analysis confirms these differences at the protein level. In addition, Dkk-3-positive colorectal cancer samples showed a statistically significantly higher mean microvessel count (9.70 vessels) than did Dkk-3-negative samples (6.82 vessels;  $P = 0.001$ ). Furthermore, it must be emphasized that the positivity rate of Dkk-3 expression increases in parallel with the microvessel count per sample ( $P < 0.0001$ ). From these cardinal findings we assume that Dkk-3 is a pro-angiogenic factor involved in neovascularization during tumor growth. Correlating Dkk-3 expression with several clinicopathological parameters including survival showed some absolutely remarkable associations, for example improved disease-free sur-

Table 1  
Dkk-3 expression in colorectal cancer tissue and clinicopathologic variables ( $n = 403$ )

Variables	All cases ( $n$ )	Dkk-3-positive ( $n = 174, 43.1\%$ )	Dkk-3-negative ( $n = 83, 20.6\%$ )	No microvessels ( $n = 146, 36.2\%$ )	Significance (P)
Gender	403				
Male	224	106 (47.3)	41 (18.3)	77 (34.4)	
Female	179	68 (38.0)	42 (23.3)	69 (38.5)	0.152
Mean age (95% CI)	69.1 (67.9-70.2)	68.0 (66.3-69.8)	67.2 (64.4-70.1)	71.3 (69.36-73.2)	0.018
Tumor site	403				
Colon	260	105 (40.3)	48 (18.5)	107 (41.2)	
Rectum	143	69 (48.3)	35 (24.5)	39 (27.2)	0.020
Tumor differentiation	403				
Grade 1/2	332	149 (44.0)	71 (21.4)	112 (33.7)	
Grade 3/4	71	25 (35.2)	12 (16.9)	34 (47.9)	0.079
pT stage	403				
pT1	37	15 (40.5)	13 (35.1)	9 (24.4)	
pT2	73	39 (53.4)	12 (16.4)	22 (30.2)	
pT3	247	105 (42.5)	50 (20.2)	92 (37.3)	
pT4	46	15 (32.6)	8 (17.4)	23 (50.0)	0.054
pN stage	387				
pN-	192	91 (47.4)	40 (20.8)	61 (31.8)	
pN+	195	79 (40.5)	39 (20.0)	77 (39.5)	0.260
M stage	403				
M0	319	137 (42.9)	71 (22.3)	111 (34.8)	
M1	84	37 (44.0)	12 (14.3)	35 (41.7)	0.230
Tumor progression	403				
No	260	109 (42.0)	62 (23.8)	89 (34.2)	
Yes	143	65 (45.5)	21 (14.7)	57 (39.8)	0.089

Table 2  
Univariate analysis of disease-free survival (colorectal cancer tissue;  $n = 403$ )

Variable	$\chi^2$	DF	P
Gender (Male vs. Female)	1.06	1	n.s.
Age (continuous)	0.45	1	n.s.
Tumor site (Colon vs. Rectum)	0.30	1	n.s.
Tumor differentiation (G1/G2 vs. G3/G4)	12.21	1	0.000
pT stage (pT1 vs. pT2 vs. pT3 vs. pT4)	40.38	3	0.000
pN stage (pN- vs. pN+)	72.83	1	0.000
M stage (M0 vs. M1)	357.11	1	0.000
Dkk-3 (Dkk-3-pos. vs. Dkk-3-neg. vs. no microvessels)	5.73	2	0.057

Table 3  
Multivariate analysis of disease-free survival (colorectal cancer tissue;  $n = 403$ )

Variable	RR	95% CI for mean		P
		Lower bound	Upper bound	
pN stage (pN- vs. pN+)	2.21	1.41	3.47	0.001
M stage (M0 vs. M1)	13.13	8.38	20.55	0.000
Dkk-3 no microvessels				0.69
Dkk-3-neg	1.21	0.73	2.02	0.46
Dkk-3-pos	1.06	0.64	1.76	0.82

RR . . . relative risk; CI . . . confidence interval.

vival for patients with Dkk-3-negative samples ( $P = 0.057$ ). Nevertheless, it is difficult to interpret these clinical results because of the significant proportion of samples without microvessels ( $n = 146; 36.2\%$ ).

It is widely accepted that the "angiogenic switch" is "on" if the balance between pro-angiogenic and anti-angiogenic factors is tipped in favor of angiogene-

sis [27,28]. Pro- and anti-angiogenic molecules can emanate from cancer cells, cancer stromal cells, blood and the extracellular matrix [29]. Sugiyama et al. used laser capture microdissection in tissue samples from 11 patients undergoing colorectal resection for cancer to isolate pure populations of cancer cells, cancer stromal cells (including vascular endothelial cells) and corre-

sponding adjacent normal epithelial and stromal cells for gene expression arrays. They demonstrated more genes contributing to angiogenesis in cancer cells than in adjacent normal epithelial cells and more in cancer stromal cells than in adjacent normal stromal cells. In addition, expression of the angiogenesis-related proteins VEGF and thrombospondin 2 was determined using immunohistochemistry. Both proteins were stained strongly positive to positive in cancer cells and cancer stromal cells, but also positive in adjacent normal stromal cells [30]. Our study illustrates that colorectal adjacent non-cancerous tissue is similar in comparison to colorectal cancer tissue with regard to Dkk-3 protein expression: 1) We found no positive staining reaction for Dkk-3 in the glandular epithelium of 318 colorectal adjacent non-cancerous tissue samples; 2) a positive staining reaction for Dkk-3 was seen in endothelial cells of all microvessels in 150/268 (56%) CD31-positive samples; 3) a significantly higher mean microvessel count (14.51 vessels) was shown in Dkk-3-positive samples than in Dkk-3-negative samples (6.64 vessels;  $P < 0.0001$ ); 4) Dkk-3 expression increased with increasing number of microvessels per sample ( $P < 0.0001$ ). These results support the concept that carcinogenesis is a multicellular process in which alteration of the surrounding microenvironment is required for tumor progression [31,32]. In our opinion, scientific focus must return to the "entire" tumor, which includes the epithelial cancer cells, the surrounding cancer stromal cells, but also the adjacent non-cancerous tissue with its altered vascular endothelial cells or abnormal microvessels. As a result of various angiogenic factors secreted mainly by cancer cells and tumor-associated stromal cells, more than half of colorectal carcinomas are characterized by intensified angiogenic activity, a feature related to unfavorable outcome [33].

In conclusion, our study demonstrates for the first time that microvessels of colorectal cancer and adjacent non-cancerous tissue are similar with regard to Dkk-3 protein expression, but distinct from normal colorectal mucosa. Therefore, Dkk-3 can be considered a putative pro-angiogenic protein in the neovascularization process. It may have the potential to serve as a marker for neoangiogenesis and might be a target structure for novel therapeutic approaches. Nevertheless, it is mandatory that the findings presented here be confirmed with routinely processed tissue sections.

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