Dickkopf-1 expression is down-regulated during the colorectal adenoma–carcinoma sequence and correlates with reduced microvessel density and VEGF expression

Zhiyong Liu,1,2,3 Baocun Sun,1,2,3,4 Lisha Qi,1,2,3 Yixian Li,4 Xiulan Zhao,4 Danfang Zhang4 & Yanhui Zhang1,2,3

1Department of Pathology, Tianjin Medical University Cancer Institute and Hospital, 2The Key Laboratory of Tianjin Cancer Prevention and Treatment, 3National Clinical Research Centre for Cancer, and 4Department of Pathology, Tianjin Medical University, Tianjin, China


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Aims: Dickkopf-1 (Dkk1), an antagonist of the Wnt–β-catenin signalling pathway, has been reported to play a role in cancer progression. However, little is known about the role of Dkk1 during the colorectal adenoma–carcinoma sequence. This study aimed to elucidate the role of Dkk1 in tumorigenesis and angiogenesis in colorectal cancer.

Methods and results: We examined Dkk1 expression immunohistochemically in 476 colorectal tissue samples, including 46 sets of matched specimens. Dkk1 expression was down-regulated during the colorectal adenoma–carcinoma sequence, both among the 476 samples and in the 46 sets of matched specimens. Dkk1 expression was correlated with decreased microvessel density (P < 0.05) and VEGF expression.

Conclusions: This study is the first to show the roles of Dkk1 during the colorectal adenoma–carcinoma sequence, which may involve suppression of the tumorigenesis and angiogenesis of CRC. Dkk1 could therefore serve as a potential target for tumour therapy.

Keywords: adenoma–carcinoma sequence, angiogenesis, colorectal cancer, Dickkopf-1, tumorigenesis

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, and is the fourth most common cause of cancer-related death.1 Most CRCs undergo a series of changes from normal to dysplastic epithelium to carcinoma, accompanied by the accumulation of multiple genetic alterations, known as the adenoma–carcinoma sequence.2–5 This process makes CRC an excellent system for studying genetic alterations and the manner in which they affect tumour progression. There is no doubt that angiogenesis plays an important role in tumour progression.6 Thus, characterizing the deeper molecular events involved in the dynamic angiogenesis changes during...
the adenoma–carcinoma sequence of CRC would help to improve identification of potential therapeutic targets.

The Wnt–β-catenin signalling pathway controls diverse fundamental biological processes.7,8 Activating mutations of the Wnt signalling pathway have a well-established role in ~90% of CRCs, but are less frequent in other cancers.9–12 In addition to direct effects on tumour cells, the Wnt–β-catenin pathway has been implicated in regulation of the vascular system.13

Dickkopf-1 (Dkk1) is a secreted protein that antagonizes the Wnt–β-catenin signalling pathway. Dkk1 can sequester lipoprotein receptor-related protein 5 (LRP5)/LRP6 away from the Frizzled–LRP6 complex by binding to the LRP5/LRP6 component of the Wnt receptor complex, thereby inhibiting TCF/LEF transcription and canonical Wnt signalling. Dkk1 not only plays essential roles in vertebrate development, but also acts as a tumour suppressor in CRC, renal cell carcinoma, breast cancer, and melanoma.14–20 Our previous study showed that Dkk1 expression is inversely correlated with tumour stage and the presence of metastasis and recurrence, and also inhibits the epithelial–mesenchymal transition of colon cancer cells and contributes to colon cancer suppression.21 Huang et al.22 found that Dkk1 could be down-regulated by lysine-specific demethylase 1 and was involved in colorectal tumorigenesis. Although these data point to a critical suppressive role for Dkk1 in CRC, the role of Dkk1 in carcinogenesis and angiogenesis in the colorectal adenoma–carcinoma sequence remains unclear.

In this study, Dkk1 expression was examined in 476 colorectal tissue samples, including 46 sets of matched specimens, and correlations between Dkk1 expression, microvessel density (MVD) and VEGF expression were analysed to explore the effect of Dkk1 on carcinogenesis and angiogenesis during the colorectal adenoma–carcinoma sequence.

Materials and methods

TISSUE SAMPLES

We obtained 476 formalin-fixed, paraffin-embedded colorectal tissue samples from the Department of Pathology at Tianjin Medical University Cancer Institute and Hospital, taken from January 2002 to December 2004. All of the cancers in the study were resection specimens. The use of the tissue samples in this study was approved by the Institutional Research Committee. The 476 tissue samples were from 349 patients, and included 263 carcinomas, 91 adenomas with low-grade dysplasia, 41 adenomas with high-grade dysplasia, and 81 samples of histologically normal mucosa which were taken >50 mm distant from the corresponding cancer or adenoma tissues. The 132 adenomas included 70 tubular adenomas, 47 tubulo-villous adenomas, and 15 villous adenomas. Of the 263 cancer samples, 15 were stage I, 158 were stage II, 73 were stage III, and 17 were stage IV. Ninety colon cancer specimens were from patients with metastasis and 14 from patients with recurrence. The detailed clinical characteristics of the 349 cases from which samples were obtained are shown Tables 1 and 2.

None of the patients received chemotherapy or radiotherapy before surgery. Among the 476 colorectal tissue samples, 46 were matched samples (histologically normal mucosa, adenomas, and carcinomas) obtained from one operation for each patient. All of the samples were subjected to a uniform protocol for fixation/dissection and processing. The histopathological type and grade of the sections from all cases were reviewed by two senior pathologists, and confirmed by a third pathologist who specialized in colorectal pathology, according to the World Health Organization’s 2004 classification.

CELLS AND REAGENTS

Antibodies against Dkk1, CD34 and VEGF were from Santa Cruz Biotechnology (Santa Cruz, CA, USA),
and secondary HRP-conjugated antibodies from Zhongshan Chemical Co. (Beijing, China). The human CRC cell line HCT116 and human umbilical vein endothelial cells (HUVECs) were obtained from the Cell Resource Centre, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College (Beijing, China). Fetal bovine serum (FBS) was from Hyclone (South Logan, UT, USA). Plasmids containing the human full-length combinational DNA fragment of Dkk1 were from Genechem Technology (Shanghai, China). Matrigel was from BD Biosciences (San Jose, CA, USA). BALB/C nude mice (aged 4–5 weeks) were obtained from Wei Tong Li Hua Experimental Animal Co. (Beijing, China).

TISSUE IMMUNOHISTOCHEMICAL ANALYSIS

According to the manufacturer’s instructions, the sections were incubated overnight at 4°C with the primary specific antibody (Dkk1, 1:50; CD34, 1:25; and VEGF, 1:50), and immunostained using the secondary HRP-conjugated antibody. Signals were revealed using 3,3′-diaminobenzidine buffer as the substrate. The sections were scored blindly by two observers using a microscope at ×200 magnification.

The results of Dkk1 immunohistochemical analysis were interpreted according to the method described by Gao et al.23 The intensity of Dkk1 staining was graded on a scale from 0 to 3 (0 for no staining, 1 for weak immunoreactivity, 2 for moderate immunoreactivity, and 3 for strong immunoreactivity); and the percentage of immunoreactivity was also scored on a scale from 0 to 3 (0 for no positive cells, 1 for <25% positive cells, 2 for 25–50% positive cells, and 3 for >50% positive cells). The two scores were then multiplied to obtain a composite Dkk1 expression score. Dkk1 expression was classified as negative (−, score of 0), weakly positive (+, score of 1 or 2), moderately positive (+++, score of 3 or 4), or intensely positive (++++, score of 6, 7, 8, or 9).

The staining of VEGF was scored by the intensity of the immunoreaction (−, no immunoreactivity; +, weakly positive; ++, moderately positive; and ++++, intensely positive). MVD was determined by immunohistochemical staining of CD34. Ten separately located tumour areas with the highest density of discrete microvessels (hot spots) within each section were selected for quantification of blood vessels at ×200 magnification. The mean value of the vessel counts in the selected spots was retained as the final MVD value. Vessels with thick smooth muscle walls or with a diameter more than eight times the diameter of a single red blood cell were also excluded.

PLASMID TRANSFECTION

Transfection with a plasmid carrying Dkk1 and a controlled scrambled plasmid was performed with Lipofectamine 2000 according to the manufacturer’s instructions. G418-resistant cells were screened to establish stable HCT116 cells that overexpressed Dkk1.

Table 2. Dkk1 expression during the colorectal adenoma–carcinoma sequence

<table>
<thead>
<tr>
<th>Dkk1 expression, n (%)</th>
<th>Total</th>
<th>−</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>χ²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All specimens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histologically normal mucosa</td>
<td>81</td>
<td>14 (17.3)</td>
<td>21 (25.9)</td>
<td>43 (53.1)</td>
<td>3 (3.7)</td>
<td>23.03</td>
<td>0.006*</td>
</tr>
<tr>
<td>Adenoma with low-grade dysplasia</td>
<td>91</td>
<td>22 (24.2)</td>
<td>21 (23.1)</td>
<td>31 (34.0)</td>
<td>17 (18.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoma with high-grade dysplasia</td>
<td>41</td>
<td>13 (31.7)</td>
<td>10 (24.4)</td>
<td>16 (39.0)</td>
<td>2 (4.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>263</td>
<td>39 (14.8)</td>
<td>69 (26.2)</td>
<td>111 (42.2)</td>
<td>44 (16.7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Matched specimens      |       |   |   |    |     |    |         |
| Normal                 | 46    | 2 (4.3) | 7 (15.2) | 20 (43.5) | 17 (37.0) | 29.00 | <0.001**|
| Adenoma                | 46    | 8 (17.4) | 11 (23.9) | 20 (43.5) | 7 (15.2)   |
| Carcinoma              | 46    | 17 (37) | 14 (30.4) | 12 (26.1) | 3 (6.5)    |

*P < 0.05; **P < 0.001.
**Western Blot Analysis**

The whole-cell lysates were prepared for immunoblotting. Proteins were resolved by SDS-PAGE and analysed by western blotting using PVDF membranes (Millipore, Temecula, CA, USA). Thirty-five micrograms of protein was loaded per well for Dkk1 western blotting, and 45 μg of protein per well for VEGF western blotting. Blots were blocked and incubated with primary antibodies (Dkk1, 1:100; and VEGF, 1:100) overnight at 4°C. They were then washed in TBS containing 0.1% Tween-20, and labelled with goat anti-mouse IgG–HRP (1:5000; Santa Cruz Biotechnology) or goat anti-rabbit IgG–HRP (1:5000; Santa Cruz Biotechnology). Equal loading of samples was confirmed by probing the membranes with β-actin antibody.

**In-Vitro 3D Coculture Experiments**

The 3D coculture system was used to determine whether Dkk1 secreted by CRC cells can induce endothelial cells to form tube-like structures. The upper surface of a chamber filter (3 μm pore size) of a 12-well tissue culture plate (BD Biosciences) was coated with 100 μl of Matrigel at a final concentration of 1.5 mg/ml. The HUVECs were trypsinized, suspended in the complete medium at 1 × 10^5 cells/ml, and plated onto the surface of the Matrigel at 800 μl/well. Then, 1 × 10^5 Dkk1-overexpressing HCT116 cells or control HCT116 cells were added in 2 ml of medium to the lower chamber and incubated at 37°C for 48 h.

**In-Vivo Assay**

Twenty mice were randomly divided into two groups, and received either 3 × 10^6 control HCT116 cells (transfected with a controlled scrambled plasmid) or 3 × 10^6 HCT116 cells overexpressing Dkk1 (clone 5) by subcutaneous injection in the right groin. All mice were killed 21 days after injection. After the body weight had been recorded, the tumours were excised from each mouse, fixed in formalin, and embedded in paraffin. The tissues were then subjected to H&E and immunohistochemical staining.

**Statistical Analysis**

SPSS v.16.0 (SPSS, Chicago, IL, USA) was used for data analysis. Differences in Dkk1 and VEGF immunohistochemical staining and MVD between groups were compared by use of χ^2 or Fisher’s exact tests in human samples. The correlation between Dkk1 expression and the colorectal adenoma–carcinoma sequence was evaluated by calculating the Spearman rank correlation coefficient. Two-tailed P-values of <0.05 were considered to be statistically significant.

**Results**

**Dkk1 Expression is Down-Regulated During the Colorectal Adenoma–Carcinoma Sequence**

We observed significant differences in Dkk1 expression during the colorectal adenoma–carcinoma sequence in 476 colorectal tissue specimens (P = 0.006; Table 2). Histologically normal mucosa tissue samples showed the most intense immunostaining for Dkk1 (Figure 1Aa,b). Dkk1 was strongly expressed in adenomas with low-grade dysplasia (Figure 1Ac,d), whereas less intense immunostaining was observed in adenomas with high-grade dysplasia (Figure 1Ae,f). Most carcinomas showed an absence or weak expression of Dkk1 (Figure 1Ag,h). Dkk1 expression was also analysed in the 46 sets of matched specimens (including histologically normal mucosa, adenomas, and carcinomas) obtained from one operation from each patient, to obtain a more defined picture of Dkk1 expression in the adenoma–carcinoma sequence. Table 2 and Figure 1B show the consistent down-regulation of Dkk1 expression from normal mucosa to adenoma to carcinoma within individual patients. Furthermore, a negative relationship was found between Dkk1 expression and the adenoma–carcinoma sequence (r_s = −0.446, P < 0.001) in these 46 sets of matched samples.

**Dkk1 Levels are Associated with MVD Counts and VEGF Expression in the Adenoma–Carcinoma Sequence**

CD34 and VEGF expression in the 46 sets of matched specimens was analysed to assess the relationship between Dkk1 and angiogenesis in the adenoma–carcinoma sequence of CRC. The median MVD was 9.02 (range: 1.20–33.80). The 138 samples stained with CD34 comprised 71 low-MVD cases (microvessel counts below the median) and 67 high-MVD cases (microvessel counts above the median). Twenty (74.1%) had high MVD among all 27 cases of Dkk1-negative samples, as shown in Table 3 and Figure 2.
The high MVD ratios decreased as the grade of Dkk1 expression level became strong (53.1%, 46.2% and 22.2% in the Dkk1+, Dkk1++ and Dkk1++++ groups, respectively).

Additionally, Dkk1 expression was significantly lower in the samples with moderately and strongly positive VEGF expression than in those with negative or weakly positive VEGF expression (Table 3; Figure 2). A negative relationship was found between Dkk1 expression score and MVD ($r_s = -0.317, P < 0.001$). A negative relationship was also found between Dkk1 and VEGF expression scores ($r_s = -0.310, P < 0.001$). These findings suggest that Dkk1 has an inhibitory effect on tumour angiogenesis in the adenoma–carcinoma sequence.

Table 3. Correlations between expression of Dkk-1 and MVD and VEGF expression

<table>
<thead>
<tr>
<th>Dkk1 expression</th>
<th>MVD expression, n (%)</th>
<th>VEGF expression, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Low</td>
</tr>
<tr>
<td>--</td>
<td>27</td>
<td>7</td>
</tr>
<tr>
<td>+</td>
<td>32</td>
<td>15</td>
</tr>
<tr>
<td>++</td>
<td>52</td>
<td>28</td>
</tr>
<tr>
<td>+++</td>
<td>27</td>
<td>21</td>
</tr>
</tbody>
</table>

* $P < 0.05$.

**Table 3. Correlations between expression of Dkk-1 and MVD and VEGF expression**

Up-regulated Dkk1 expression was confirmed by western blotting (Figure 3A) after HCT116 colon cancer cells stably overexpressing Dkk1 had been established. As compared with the control HCT116 cells, Dkk1-overexpressing HCT116 cells had down-regulated VEGF expression (Figure 3B).

Given that Dkk1 is a secreted protein, HUVECs were cocultured with Dkk1-overexpressing HCT116 cells and controls. At 3 h after seeding, the HUVECs cocultured with the control cells had a tendency to...
form tube-like structures, whereas those cocultured with Dkk1-overexpressing HCT116 cells failed to form tube-like structures (Figure 3C). At 20 h after seeding, the tube-like structures formed by HUVECs cocultured with the control cells increased in number as compared with the tube-like structures formed by HUVECs cocultured with Dkk1-overexpressing HCT116 cells (35.20 ± 6.53 versus 22.80 ± 3.70, P < 0.05), and became more intact (Figure 3C).

DKK1 INHIBITS IN-VIVO TUMOUR GROWTH AND POTENTIALLY REDUCES ANGIOGENESIS IN ANIMAL MODELS

Dkk1-overexpressing cells grew into smaller tumour masses than control cells (Figure 4A,B). MVDs were determined by immunohistochemical staining of CD34. In agreement with in-vitro findings, tumour sections from Dkk1-transfected HCT116 cells showed markedly lower MVD counts (7.26 ± 1.50 versus 12.72 ± 3.67, P < 0.05) and lower VEGF expression than those of sections from control cells (Figure 4C), further indicating the anti-angiogenesis effect of Dkk1 on CRC.

Discussion

Dkk1 expression has been shown to be down-regulated in CRC. However, no study has been conducted to analyse Dkk1 expression in the colorectal adenoma–carcinoma sequence with a sample size large enough to explore the effect of Dkk1 on tumorigenesis. In our study, Dkk1 immunohistochemical staining of 476 colorectal tissue specimens revealed a negative correlation between Dkk1 expression and the colorectal adenoma–carcinoma sequence. We also observed the same relationship between Dkk1 expression and the adenoma–carcinoma sequence in 46 sets.
of matched colorectal specimens, in which the influence of other factors (especially those associated with heterogeneity among individuals) was reduced.

Another key observation was the involvement of Dkk1 in the regulation of angiogenesis during adenoma–carcinoma progression. We found that Dkk1 expression was negatively correlated with MVD and VEGF expression in the CRC tissues. The in-vivo coculture experiment also indicated that Dkk1 secreted by HCT116 cells could impair HUVEC tube-like structure formation. The Dkk1-overexpressing HCT116 cell tumour xenograft in nude mice showed a dramatic decrease in MVD accompanied by a significant decrease in VEGF level. To our knowledge, the paracrine anti-angiogenesis effect of Dkk1 was first reported in CRC.

Previous studies have shown that Wnt–β-catenin signalling is active in endothelial cells, and plays an
important role in vascular morphogenesis in the embryo and in organ-specific endothelial cell differentiation.\textsuperscript{13,26} Altered Wnt–β-catenin signalling may influence proper endothelial cell function and contribute to several vascular defects.\textsuperscript{27–29} In this study, we showed that the Wnt–β-catenin signalling antagonist Dkk1 inhibited tube formation \textit{in vitro} and down-regulated the expression of VEGF, a cytokine that is considered to be a key regulator of angiogenesis. Interestingly, cross-talk has been found between the Wnt–β-catenin signalling pathway and VEGF.\textsuperscript{30} Activated Wnt–β-catenin signalling induced VEGF synthesis in colonic neoplasia and increased angiogenesis.\textsuperscript{31} Our previous study demonstrated that Dkk1 overexpression decreased the expression and intracellular distribution of β-catenin, and the expression of the Wnt–β-catenin signalling pathway target proteins c-myc and cyclin D1, accounting for the low levels of Wnt–β-catenin signalling activity.\textsuperscript{21} Therefore, the anti-angiogenesis effect caused by Dkk1 in CRC may have been at least partially, caused by removal of the inhibitory effect of Dkk1 on the Wnt–β-catenin pathway.

However, reports on the role of Dkk1 in angiogenesis and tumour progression are contradictory or inconsistent. One study showed that Dkk1 enhanced endothelial colony-forming cell proliferation and the formation of pseudotubes in Matrigel.\textsuperscript{32} Yu et al.\textsuperscript{13} observed that elevated Dkk1 expression was associated with cytoplasmic/nuclear β-catenin accumulation and a poor prognosis in hepatocellular carcinomas. Takahashi et al.\textsuperscript{14} found that Dkk1 was overexpressed in pancreatic adenocarcinoma cells and was involved in tumour invasion. Therefore, the effects of Dkk1 on the carcinogenesis and angiogenesis of tumours are probably tumour type-dependent and influenced by multiple mechanisms. Further studies are needed to further elucidate the function and mechanisms of Dkk1 in tumour development.

Acknowledgements

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Conflict of interest

The authors state that they have no conflict of interest.

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