



DKK4 is important in Snail1-induced chemoresistance to fluorouracil in colorectal cancer

Wenbin Shen^{1,2}, Long Cui^{1,2}, Wei Chen^{1,2}

¹Department of Colorectal and Anal Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200032, China;

²Shanghai Colorectal Cancer Research Center, Shanghai 200032, China

Contributions: (I) Conception and design: All authors; (II) Administrative support: Cui, W Chen; (III) Provision of study materials or patients: Cui, W Chen; (IV) Collection and assembly of data: W Shen; (V) Data analysis and interpretation: W Shen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Wei Chen. 1665 Kongjiang Rd, Shanghai 200032, China. Email: weichendr@126.com; Long Cui. 1665 Kongjiang Rd, Shanghai 200032, China. Email: longcuidr@126.com.

Background: The overexpression of Snail1 is correlated with the epithelial-mesenchymal transition (EMT) in many cancers. However, the correlation between Snail1 and chemoresistance in colorectal cancer (CRC) as well as the underlying mechanism remain unknown. In this study, we evaluated the relationship between Snail1 and chemoresistance in CRC and determined whether DKK4 was involved in Snail1-induced chemoresistance.

Methods: IHC was performed to determine the correlation between Snail1 and DKK4 and their relationship to chemoresistance in CRC. Additionally, a cellular functional assay was performed to study the DKK4 expression regulated by Snail1 in CRC cell lines.

Results: In our cohort, a high level of Snail1 indicated chemoresistance and poor prognosis. Furthermore, a strong correlation between DKK4 and Snail1 was observed in our CRC samples. Knockdown of Snail1 in LoVo cells resulted in the downregulation of DKK4, whereas the overexpression of Snail1 in CRC cell lines resulted in resistance to 5-fluorouracil (5-FU) treatment, with an up-regulation of the DKK4 level. Knockdown of DKK4 by siRNA in Snail1-overexpressing CRC cell lines reversed the resistance to 5-FU treatment induced by Snail1 overexpression.

Conclusions: Taken together, our results indicated that DKK4 could be a downstream gene that is regulated by Snail1 and could play an important role in Snail1-induced CRC chemoresistance to 5-FU. Targeting downstream Snail1 genes may provide a new therapeutic strategy for the treatment of advanced CRC.

Keywords: Snail1; DKK4; colorectal cancer (CRC); chemoresistance; 5-fluorouracil (5-FU)

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide (1). In clinical, the prognostic assessment of CRC is traditionally based on the TNM stage. The advanced the TNM stage is correlated with the poorer prognosis. In addition to the TNM stage, the level

of CEA, tumor histopathological grade and other factors like KRAS, PIK3A mutations all influence the prognosis. Currently, molecular classification of CRC is a promising prognostic system which requires a deeper understanding of the molecular mechanism of tumor initiation, progression, invasion and resistance to chemotherapy (2,3).

5-fluorouracil (5-FU) has been the most frequently used chemotherapy for advanced CRC for the last 40 years. Despite the widespread use of the 5-FU, resistance to 5-FU-based chemotherapy remains a major problem in the treatment of CRC (4). Although the prognosis for patients with advanced CRC has improved because of the development of novel therapeutic strategies (5), unmasking the underlying mechanism of CRC chemoresistance could lead to novel therapeutic strategies.

Snail1, a Zinc-finger transcription factor family member, is one of the most important EMT related transcriptional regulators (6,7). Snail1 is overexpressed in many malignant cancers, including CRC (8). Snail1 is overexpressed in CRC, and it is inversely correlated with the E-cadherin level in CRC (8). Snail1 has also been demonstrated to have crucial roles in CRC by regulating prostaglandin E2 and vitamin D (9-11). Notably, Snail1 can suppress the expression of Vitamin D receptor (VDR) by binding to the promoter region of exon 1a of the human VDR gene (12,13). Therefore, a high level of Snail1 may be responsible for the failure of Vitamin D analogue therapy in CRC patients (10,14). The overexpression of Snail1 can promote EMT, cancer stem cell growth and metastasis, and cancer stem cells are correlated with chemoresistance in many cancers. However, the correlation between Snail1 and chemoresistance in CRC as well as the underlying mechanism of Snail1-induced chemoresistance remain unknown.

The Dickkopf (DKK) family, which consists of 4 secretory glycoproteins, namely DKK1, DKK2, DKK3, and DKK4, can suppress canonical Wnt signaling by binding to low-density lipoprotein receptor-related protein-5 (LRP5) (15,16). There is a hyper- or hypo-expression of DKK4 in many different malignant diseases (17-19), and DKK4 is reported to be correlated with chemoresistance in CRC (20,21). Because few compounds have been developed to target Snail1, uncovering Snail-regulated-genes that can be targeted by drugs could contribute to the development of new therapeutic strategies against Snail1-mediated chemoresistance. Therefore, the aim of this study was to analyze the co-expression patterns of Snail1 and DKK4 in CRC samples and their relationship to chemoresistance. Further, we tested the effects of Snail1 and DKK4 in CRC cell lines. Here, we showed that the high expression level of Snail1 was correlated with poor prognosis and chemoresistance in CRC. The overexpression of Snail1 in CRC cell lines could result in resistance to 5-FU treatment by upregulating the DKK4 level. The DKK4 expression level was correlated to Snail1 expression in

CRC. Therefore, our results indicated that a novel Snail1/DKK4 regulation axis in CRC which is involved in the chemoresistance of CRC.

Methods

Patients and tissue samples

Patients with histologically confirmed CRC who received chemotherapy after curative resection in Xinhua Hospital were included in this study. Patients who underwent neoadjuvant treatment or R1 resection were excluded from the analysis. Formalin-fixed, paraffin-embedded tumor blocks were collected. Patients with local recurrence or distant metastasis <6 months after chemotherapy were classified as having a chemoresistance response, and those with local recurrence or distant metastasis for >6 months were classified as having a chemosensitive response (22-24).

Immunohistochemistry staining and scoring

Immunohistochemical detection of Snail1 and DKK4 was performed as previously described (25). In short, the paraffin sections were treated according to a standard protocol. After incubating with the anti-Snail1 antibody and anti-DKK4 antibody overnight, sections were washed 3 times with PBS and incubated with HRP-conjugated secondary antibody (GK500710; Gene Company Ltd.) for 30 min at RT. Then, staining was developed with DAB solution for 10 seconds. The sections were counterstained with 0.1% hematoxylin and then sealed with coverslips.

IHC scoring of DKK4 was previously described (26,27). DKK4 staining in tissues was scored by the staining intensity (0-lack of staining, 1-mild staining, 2-moderate staining, and 3-strong staining), and the score was multiplied by of the percentage of staining {[1] <25%, [2] 25–50%, [3] 51–75%, and [4] >75%}, which ranged from 0-12. When the multiplication score was >6, the DKK4 expression was considered high. The methods for scoring Snail1 are described in a previous study (27). Two histopathologists were blindly assigned to review the slides and score the staining.

Cell culture

The human CRC cell lines, SW480, HCT116 and LoVo, were purchased from American Type Culture Collection(ATCC).SW480, HCT116 and LoVo were

cultured in Dulbecco's modified Eagle's medium (Hyclone) with 10% fetal bovine serum (Gibco) and 1% penicillin & streptomycin (Invitrogen).

RNAi transfection

The siRNA of Snail1 and DKK4 were purchased from Genepharma, and the sequences are listed below. In brief, si-DKK4, si-Snail1 and negative control siRNA were transfected into CRC cell lines using Lipofectamine 2000 according to the manufacturer's protocol (Thermo Fisher Scientific).

	si-1	si-2
DKK4	5'-ACGGACUGCAAUACCA-GAA-3'	5'-GGGACAGGUCUCU-CUCCAGA-3'
Snail1	5'-CAGGACUCUAAUCCA-GAGU-3'	5'-CCACUCAGAUGUCAA-GAAG-3'
Negative control	5'-UUCUCCGAACGUGU-CACGU-3'	

RNA extraction and quantitative real-time PCR

Total RNA was extracted using Trizol (TaKaRa). Complementary DNA was synthesized with PrimeScript RT-PCR Kit (TaKaRa). Quantitative RT-PCR was performed using SYBR Premix ExTaq (TaKaRa) on an ABI 7,500 real time PCR system (Applied Biosystems). The primer sequences for mRNA qRT-PCR are indicated below. The procedures for qPCR were as follows: initial denaturation at 95 °C for 1min, followed by 40 cycles of heating to 95 °C for 5 s and then at 60 °C for 34 s. The relative gene expression levels were calculated using the 2^{-ΔΔCt} analysis method.

	Forward	Reverse
DKK4	5'-GGAGCTCTGGTCCTG-GACTT-3'	5'-TCTGGTATTGCAGTC-CGTGT-3'
Snail1	5'-CTCTAGGCCCTGGCTGC-TAC-3'	5'-TCTGAGTGGGTCTG-GAGGTG-3'
GAPDH	5'-GGGCATCTTGGGCTA-CAC-3'	5'-GGTCCAGGGTTTCT-TACTCC-3'

Vector construction, retrovirus production and transduction

The full length of the Snail1 CDS region was inserted in the pBabe-puro vector by using the BamHI/EcoRI sites. In terms of the virus package, GAG and VSVG were co-

transfected into 293T cells with pBabe-puro or pBabe-Snail1-puro by using the Polyethylenimine linear (PEI, polysciences). Then, 48 to 72 hours later, virus particles were harvested for concentration. Cell lines were infected with retrovirus overnight with 1 μg/mL polybrene (Sigma) before fresh medium was added. Cells were then selected for 1 week using puromycin.

Cell viability assay

For this, 5-fluorouracil (Sigma-Aldrich) was used in our study. SW480 and HCT116 cells were seeded in 96-well plates (1.5×10⁴ for SW480 and 1×10⁴ for HCT116). Then, 24 hrs later, the cells were treated with 5-FU. After 48 hrs, 20 μL of CCK8 (Dojindo) was added to each well, and the plates were incubated at 37 °C for one and a half hours. Then, the absorbance was measured with a spectrophotometer at 450 nm.

Statistics

Statistical analyses were applied using the SPSS 19.0 software. Student's *t*-test was used for statistical analysis. Survival curves were plotted using the Kaplan-Meier method and compared with the log-rank test. The Pearson χ^2 test was used to assess the correlation between Snail1 expression and clinical parameters. Univariate and multivariate analyses were obtained using the Cox proportional hazards regression model. A two-sided P<0.05 was considered statistically significant.

Results

Increased Snail1 expression is correlated with chemoresistance and poor prognosis in CRC

Snail1 regulates cancer stem cells in human colorectal carcinoma cells (28). Stemness can lead to cancer cell resistance to the chemotherapy. We performed an immunohistochemical analysis of Snail1 in 109 paraffin-embedded CRC tissue samples (Figure 1A,B). No significant difference between the Snail1 expression level and clinicopathological features, except for gender, was observed in our cohort (Table 1). Interestingly, a high expression level of Snail1 was associated with a poor prognosis in the CRC (Figure 1C). We observed that the resistance to 5-FU-based chemotherapy was highly correlated with Snail1 (P=0.018, Table 2). As a result, the overexpression of Snail1 could be a

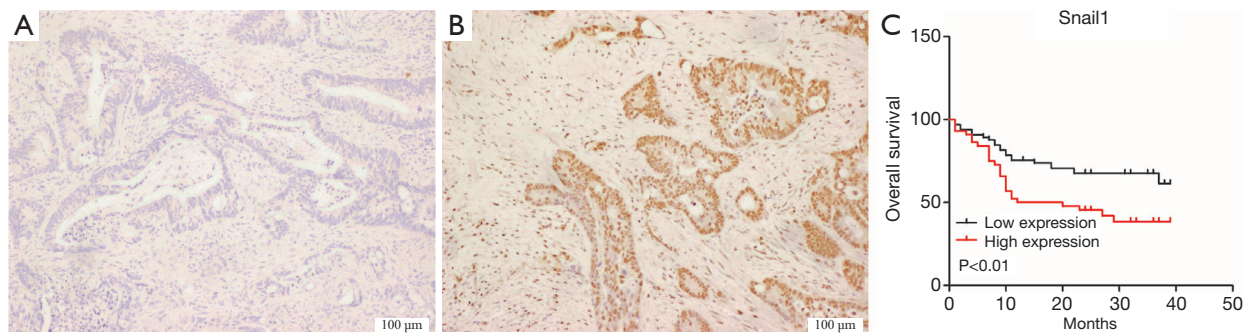


Figure 1 Prognostic value of Snail1 in colon cancer. (A,B) Immunohistochemical analysis represents low (A) and high (B) expression of Snail1 in CRC samples, respectively. (C) Kaplan-Meier plots of the overall survival of CRC patients stratified by Snail1 expression. The Log-Rank test was performed to assess the statistical significance. CRC, colorectal cancer.

Table 1 Correlation between Snail1 expression and clinicopathological characteristics (n=109)

Variable	Snail1 expression			P value
	Low	High	No. of cases	
Age, years				0.881
>50	39	57	96	
≤50	5	8	13	
Gender				0.006
Male	32	30	62	
Female	12	35	47	
Depth of invasion				0.27
pT1	0	2	2	
pT2	1	6	7	
pT3	18	27	45	
pT4	25	30	55	
Lymph node metastasis				0.092
pN0	17	36	53	
pN1	16	22	38	
pN2	11	7	18	
Distant metastasis				0.177
M0	31	53	84	
M1	13	12	25	

Table 2 Relationship between Snail1 expression and chemoresistance in CRC patients (n=109)

Effect	Snail1 expression		P value
	Low	High	
Resistance	18	22	–
Sensitive	47	22	–
Total	65	44	0.018

CRC, colorectal cancer.

Table 3 Univariate analysis of the prognostic factors for overall survival

Variables	HR	95% CI	P value
Snail1 (High vs. low)	2.680	1.504–4.775	0.001
Age (<50 vs. >50)	0.823	0.461–1.469	0.511
T3–4 vs. T1–2	1.13	0.404–3.158	0.816
Lymph node metastasis (N1–2 vs. N0)	2.919	1.562–5.455	0.001
Distant metastasis (M1 vs. M0)	11.598	5.797–23.205	0.000
Gender (Female vs. male)	0.512	0.159–1.649	0.262

potential indicator of poor prognosis and chemoresistance in CRC. Additionally, the univariate analyses indicated that Snail1 expression, lymph node metastasis and distant metastasis were correlated with the overall survival (Table 3), whereas the multi-variate analyses showed that Snail1 expression and distant metastasis were independent prognostic factors for overall survival (Table 4).

Overexpression of Snail1 in CRC cell lines leads to resistance to 5-FU treatment

To investigate the role of Snail1 in chemoresistance in CRC, Snail1 was cloned into the pBABE vector, which was utilized to generate stable cells overexpressing Snail1 in HCT116 and SW480 cells. We confirmed the overexpression of Snail1 in both cell lines using qPCR (Figure 2A). Then, the cell stably expressing Snail1 was treated with 5-FU, and the number of surviving cells was measured by CCK8.

After 48hrs of 5-FU treatment, SW480-pBABE-Snail1 and HCT116-pBABE-Snail1 cells showed a significant increase in cell survival compared to the SW480-pBABE and HCT116-pBABE vector control cells, respectively (Figure 2B,C). These results were consistent with the clinical observation of an association between Snail1 overexpression and chemoresistance in CRC.

Snail1 promotes chemoresistance by upregulating the DKK4 level in CRC

Next, we explored the underlying mechanism of Snail1-induced chemoresistance in CRC. DKK4 is reported to be involved in the chemoresistance induced by the hypermethylation of *TFAP2E* gene in CRC. Intriguingly, we found that knockdown of Snail1 in LoVo cells downregulated DKK4 mRNA level (Figure 3A) while overexpression of Snail1 had the opposite effect in both HCT116 and SW480 cells (Figure 3B). To further determine the function of DKK4 in Snail1-induced chemoresistance, two different siRNAs targeting DKK4 were used to knockdown the DKK4 level in Snail1-overexpressing cell lines. Knockdown of DKK4 was confirmed by qPCR (Figure 3C). Cells transfected with siRNA were seeded in 96-well plates and treated with different concentrations of 5-FU, and the

cell viability was measured by CCK8. Consistent with our prediction, knockdown of DKK4 in Snail1-overexpressing cell lines led to chemo-sensitivity in these cell lines (Figure 3D,E). Furthermore, a significant correlation between DKK4 and Snail1 was observed in our cohort (Figure 3F, Table 5). Taken together, our results indicated that DKK4, a vital factor in chemoresistance (29,30), is involved in Snail1-induced chemoresistance in CRC.

Discussion

Snail1, a member of Snail superfamily of transcription factors, has been implicated in many processes related to EMT and the inheritance of stem cell properties (31-33). Snail1 is regulated by LOXL2, SMAD, and HMGA2 by directly binding to the Snail1 promoter (34-36). Snail1 exerts a great role in the development of human tumors, including CRC, which is one of the most aggressive cancers (37). A high level of Snail1 in CRC was correlated with advanced stage and chemoresistance. In our cohort, we found that a high level of Snail1 in CRC patients results in resistance to 5-FU-based chemotherapy. In the CRC cell line, we also observed chemoresistance that was induced by the overexpression of Snail1. These results provide a possibility for Snail1 to act as a predictor of a response to chemotherapy.

Dysregulation of DKK4 has been frequently observed in several human malignancies, including hepatocellular carcinoma, pancreatic cancer, renal cell carcinoma and CRC (18,19). In CRC, DKK4 is involved in the chemoresistance. Induction of DKK4 expression results in the resistance to the 5-FU treatment. Transcription factor *TFAP2E* inhibits DKK4 expression in CRC cells and hypermethylation of *TFAP2E* gene is correlated with chemoresistance as well as

Table 4 Multivariate analysis of the prognostic factors for overall survival

Variables	HR	95% CI	P value
Snail1 (High vs. low)	1.960	1.043–3.685	0.037
Lymph node metastasis (N1–2 vs. N0)	1.889	0.961–3.712	0.065
Distant metastasis (M1 vs. M0)	9.346	4.456–19.602	0.000

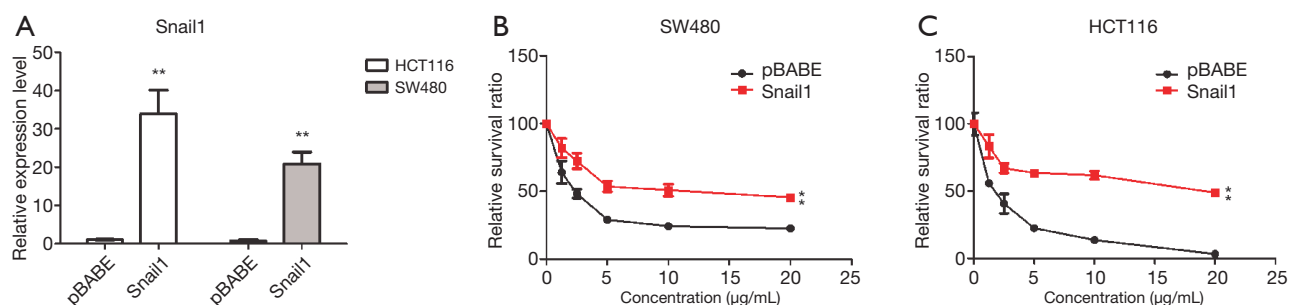


Figure 2 Increased chemoresistance to 5-FU treatment of Snail1 overexpressing cell lines. (A) qPCR analysis of the Snail1 expression level in HCT116 and SW480 cells infected with pBABE and pBABE-Snail1 viruses, respectively; (B,C) The cell response to 5-FU treatment with different concentrations was measured by CCK8.

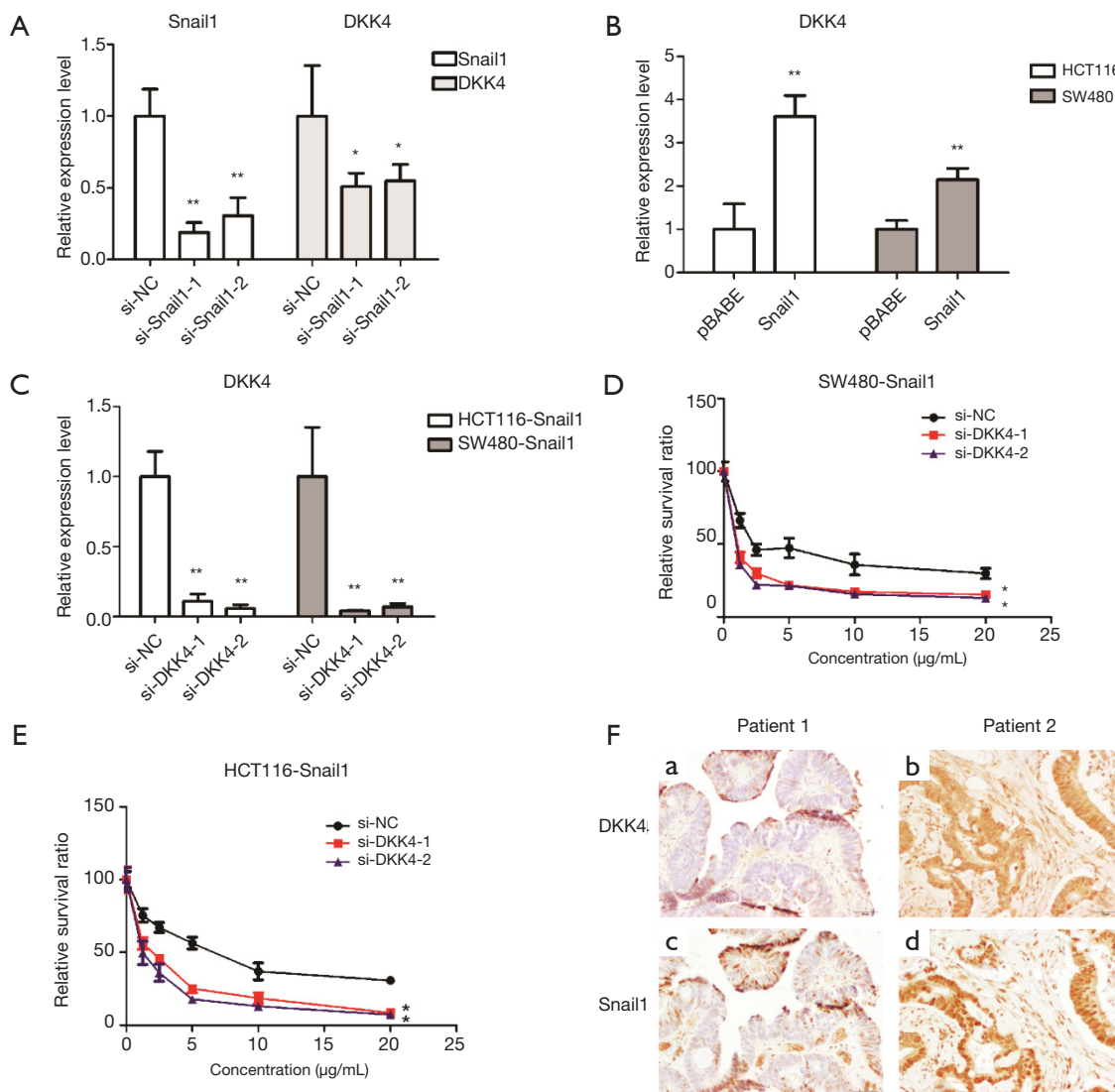


Figure 3 The expression of DKK4 is highly correlated with Snail1 *in vitro* and *in vivo*. (A) qPCR analysis of the Snail1 and DKK4 expression levels in LoVo cells that were transfected with siRNA targeting Snail1; (B) qPCR analysis of the DKK4 expression level in the HCT116 and SW480 cells that were infected with pBABE and pBABE-Snail1 viruses, respectively; (C) validation of the DKK4 knockdown efficiency in Snail1 overexpressing cell lines was carried out by qPCR; (D,E) Snail1 overexpressing cell lines were first transfected with siRNA targeting DKK4, and they were then treated with increasing concentrations of 5-FU. Cell growth was analyzed by CKK8; (F) the correlation between Snail1 and DKK4 expression was detected in the 109 CRC samples using IHC; (a) and (c) represent low expression levels of DKK4 and Snail1, respectively, and (b) and (d) represent high expression (Scale bar: 50 µm).

increased expression of DKK4 in the CRC (20,21,38). Here, our results demonstrate that the up-regulation of DKK4 by Snail1 in CRC led to chemoresistance in the CRC cell lines. Furthermore, a high level of DKK4 was not only associated with poor response to chemotherapy, but DKK4 level is positively correlated with Snail1 in our cohort. In CRC cell lines, knockdown of DKK4 reversed the chemoresistance

induced by Snail1. These findings suggested that DKK4 may be a potential downstream target of Snail1 and has an important role in Snail1-induced chemoresistance. Snail1 is a transcriptional co-repressor (31) but can also function as a transcriptional co-activator (39). Thus, Snail1 can promote the transcription of DKK4 directly or indirectly. The underlying mechanism need to be explored in the future.

Table 5 Relationship of the expression level between Snail1 and DKK4 in CRC patients (n=109)

Gene		Snail expression		P value
		Low	High	
DKK4	Low expression	50	21	–
	High expression	15	23	–
		65	44	0.002

CRC, colorectal cancer.

In summary, together with previous results, this study showed that Snail1 and DKK4 were frequently up-regulated in CRC patients and CRC cell lines. Additionally, the expression levels of Snail1 and DKK4 were associated with an advanced stage and chemoresistance in our cohort. In CRC cell lines, the overexpression of Snail1 not only resulted in resistance to 5-FU treatment, but it also increased the DKK4 expression level. In addition, knockdown of DKK4 in stably expressing Snail1 cell lines decreased the resistance to 5-FU that was induced by Snail1, which suggests DKK4 plays an important role in Snail1-induced chemoresistance. Because Snail1 is difficult to target by using the currently available drugs, targeting DKK4 in patients who are resistant to 5-FU may be a promising therapeutic strategy.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the Institutional Review Board of Xinhua Hospital, Shanghai Jiaotong University School of Medicine (Approval No. XHEC-D-2011-047) and written informed consent was obtained from all patients.

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