

Combined Serum DKK3 and Circulating CD133 Cells as Prognostic Biomarkers for Ovarian Cancer Patients

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Introduction: Ovarian cancer (OV) can seriously endanger women's physical and mental health. Serum DKK3 has been used for the diagnosis and prognosis of patients with ovarian cancer. However, the specificity of antibodies may lead to errors in the detection of plasma protein.

Methods: Circulating CD133⁺ cells from blood samples were separated by magnetic microbeads. Serum DKK3 levels were determined by ELISA. The roles of DKK3 in OV cells were analyzed in vitro.

Results: In this study, we found that the CD133⁺ subpopulation in circulating tumor cells can indicate the overall survival rate of OV patients. Serum DKK3 levels were negatively correlated with the number of circulating CD133⁺ cells in OV patients. In addition, we confirmed the inhibitory effect of recombinant human DKK3 (rhDKK3) on OV cells via reversal of the epithelial-mesenchymal transition (EMT) process.

Conclusion: Both serum DKK3 levels and circulating CD133⁺ tumor cells can be used as prognostic markers for patients with ovarian cancer.

Keywords: DKK3, ovarian cancer, CD133, Wnt, prognosis

Introduction

Many methods have been used for the clinical diagnosis of cancer.¹ Tissue biopsy is more reliable than other methods, but it carries a high risk for patients.¹ Some imaging examination methods (eg, CT and MRI) have the advantages of non-invasive and accurate positioning; however, the evaluation of the results mainly depends on clinical experience and anatomical knowledge.^{2,3} Therefore, serum markers are suitable for improving the diagnostic efficiency and safety for cancer patients.

Circulating tumor cells (CTCs) are all kinds of tumor cells existing in peripheral blood.⁴ Tumor cells invade the blood and lymphatic system, form CTCs, and are transported to the distal tissue.⁵ The early detection of CTCs in the blood has significance in making prognostic judgments, evaluation of the curative effect, and individualized treatment.⁶ CD133 is a glycoprotein expressed on hematopoietic progenitors and has been widely used in sorting cancer stem cells (CSCs).⁷ Circulating CD133⁺ cells are prognostic and diagnostic markers for colorectal cancer and gastric cancer.^{8,9}

The DKK family consists of DKK1, DKK2, DKK3, and DKK4, which modulate the Wnt signaling pathway.¹⁰ Abnormal expression of DKK1, DKK2, DKK3, or DKK4 proteins leads to malignant tumors.¹¹ The Wnt signaling pathway is

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a basic pathway involved in embryonic development and adult tissue homeostasis.¹² Secretory inhibitors, such as DKK3, inhibit the Wnt signaling pathway by competitively binding to the LRP5/6 receptor.¹³ Epithelial ovarian cancer (EOC) is the leading cause of death among all gynecological cancers.¹⁴ Serum DKK3 is a diagnostic marker for ovarian cancer.¹⁵ In this study, we compared the serum levels of DKK3 with the numbers of CD133⁺ cells in ovarian cancer (OV) patients and healthy volunteers. We found that serum DKK3 and circulating CD133⁺ cells can be used as prognostic markers for patients with ovarian cancer.

Materials and Methods

Bioinformatic Analysis

Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancer-pku.cn/>) is a newly developed interactive server to analyze cancer and normal gene expression profiling based on data from TCGA (<https://tcga-data.nci.nih.gov/tcga/>) and the Genotype-Tissue Expression project (<https://www.gtexportal.org/home/index.html>).¹⁶ We explored the relative expression of DKK1, DKK2, DKK3, and DKK4 via GEPIA based on the clinicopathological features of OV. The mutations and copy numbers of DKK3 in all kinds of cancer patients were calculated using the cBioPortal online database (www.cbioportal.org).¹⁷

Blood Samples

Blood samples were obtained from 167 patients who had not received any chemotherapy or radiotherapy prior to complete resection at the Department of Gynaecology, Shenyang Women's and Children's Hospital, between July 2010 and July 2015. All patients provided written informed consent. Control blood samples were obtained from 40 healthy volunteers. The volunteers were female and of similar ages to the patients. This study was conducted according to the principles expressed in the Helsinki Declaration of 1975 and approved by the Ethics Committee of Liaoning Medical University.

Serum DKK3 Levels

Enzyme-linked immunosorbent assay (ELISA) was used to detect the DKK3 levels in OV patients. The human DKK3 ELISA kit was purchased from R&D Systems (Shanghai, China). According to the manufacturer's protocol, the optical density was measured at 450 nm.

Sorting and Identify Circulating CD133⁺ Cells

As in our previous method, circulating CD133⁺ cells from blood samples were separated by magnetic microbeads.⁹ The purity of sorted cells was evaluated using flow cytometry (Becton Dickinson, San Jose, CA). Cells were stained with fluorescently labeled CD133-FITC (Thermo Fisher, Shanghai, China) to identify CD133 expression and DAPI to identify nucleated cells.

MTT Assay

Recombinant human DKK3 (rhDKK3) was purchased from R&D Systems (Shanghai, China). CD133⁺ cells or CD133⁻ cells were plated in 96-well plates (1000 cells per well). After 24 h, cells were treated with various concentrations of rhDKK3 (eg, 0, 5, 10, 15, 20, 25, 30 µg/mL for each). The MTT Cell Proliferation and Cytotoxicity Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China). At the end of the treatment period (48 h), 20 µL of MTT solution was added to each well. After incubation for 4 h at 37°C, absorbance density values were checked at 570 nm to determine the cell viability.

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was used to measure the changes in epithelial-mesenchymal transition (EMT). Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Shanghai, China). cDNA was synthesized using the PrimeScript™ RT Reagent Kit (Takara, Dalian, China). qPCR was carried out using the SYBR Green PCR Mix Kit (Takara) according to the instructions. The results were analyzed using the $\Delta\Delta CT$ (cycle threshold) method. The primers used in this study are summarized in Table 1.

Western Blot Analysis

Cells were washed once with PBS, and lysed for 30 min in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40) containing protease inhibitors (Cocktail; Roche, Basel, Switzerland) and phosphatase inhibitors (1 mM NaF and 1 mM Na₃VO₄). The lysates were centrifuged at 15,000×g at 4°C for 15 min. Total cell lysate proteins were separated using 10% SDS-PAGE and transferred to nitrocellulose (NC) membranes (KeyGen, Nanjing, China). The NC membranes were incubated with primary antibodies overnight at 4°C, including E-cadherin (sc-8426), N-cadherin (sc-59,987), Vimentin (sc-6260), Snail (sc-271,977), and GAPDH

Table I Relationship Between Serum DKK3 or Circulating CD133⁺ Tumor Cells and Clinicopathological Parameters of OV Patients

Clinicopathological Features	sDKK3					CD133 ⁺			
	n	Low	High	χ^2	P	≤3	>3	χ^2	P
Age (years)				13.4	0.00025			0.13	0.713
<50	83	44	39			14	69		
≥50	84	67	17			16	68		
Venous invasion				7.93	0.0048			16.1	0.00006
–	73	40	33			23	50		
+	94	71	23			7	87		
Lymphatic invasion				4.09	0.0431			0.15	0.690
–	89	53	36			15	74		
+	78	58	20			15	63		
Preoperative CA125 (U/mL)				0.88	0.3471			2.31	0.128
<600	65	46	19			8	57		
≥600	102	65	37			22	80		
Histological grade				2.25	0.324			1.52	0.468
Grade 1	44	33	11			8	36		
Grade 2	81	50	31			12	69		
Grade 3	42	28	14			10	32		
Residual tumor				0.14	0.700			5.15	0.0231
<1 cm	80	52	28			20	60		
≥1 cm	87	59	28			10	77		
FIGO stage				2.80	0.423			11.38	0.0098
I	27	19	8			10	17		
II	48	35	13			8	40		
III	68	44	24			6	62		
IV	24	13	11			6	18		

Abbreviation: χ^2 , Chi-squared distribution.

(sc-47,724) (Santa Cruz, CA, USA). Each specific antibody binding was detected with horseradish peroxidase (HRP)-conjugated respective secondary antibodies (Beyotime Biotechnology, Beijing, China). Bands were visualized using the ECL Western Blotting Analysis System (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

Statistical evaluation was performed using the Spearman correlation test to analyze rank data and using the Mann–Whitney *U*-test and Student's *t*-test to differentiate the means of the different groups. A Kaplan–Meier survival analysis was performed to obtain the survival curves. *P*-values of <0.05 were considered to indicate statistical significance. GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used to analyze all data.

Results

DKK1, *DKK2*, *DKK3*, and *DKK4* in OV Tissues

Based on the GEPIA analysis of cancer vs normal tissue, we found that *DKK3* mRNA was significantly lower in the OV tissues than that in normal tissues (Figure 1A). *DKK1*, *DKK2*, and *DKK4* showed no significant difference in OV tissues and normal tissues (Figure 1A). We also did not find any differences in *DKK1*, *DKK2*, *DKK3*, or *DKK4* mRNA in different stages of OV (Figure 1B). Kaplan–Meier analysis revealed that *DKK1*, *DKK2*, *DKK3*, and *DKK4* mRNA were not correlated with the overall survival rate or the disease-free survival rate of OV patients (*P*>0.05) (Figure 1C and D). Genetic alteration rates for OV patients were less than 2% (Figure 1E).

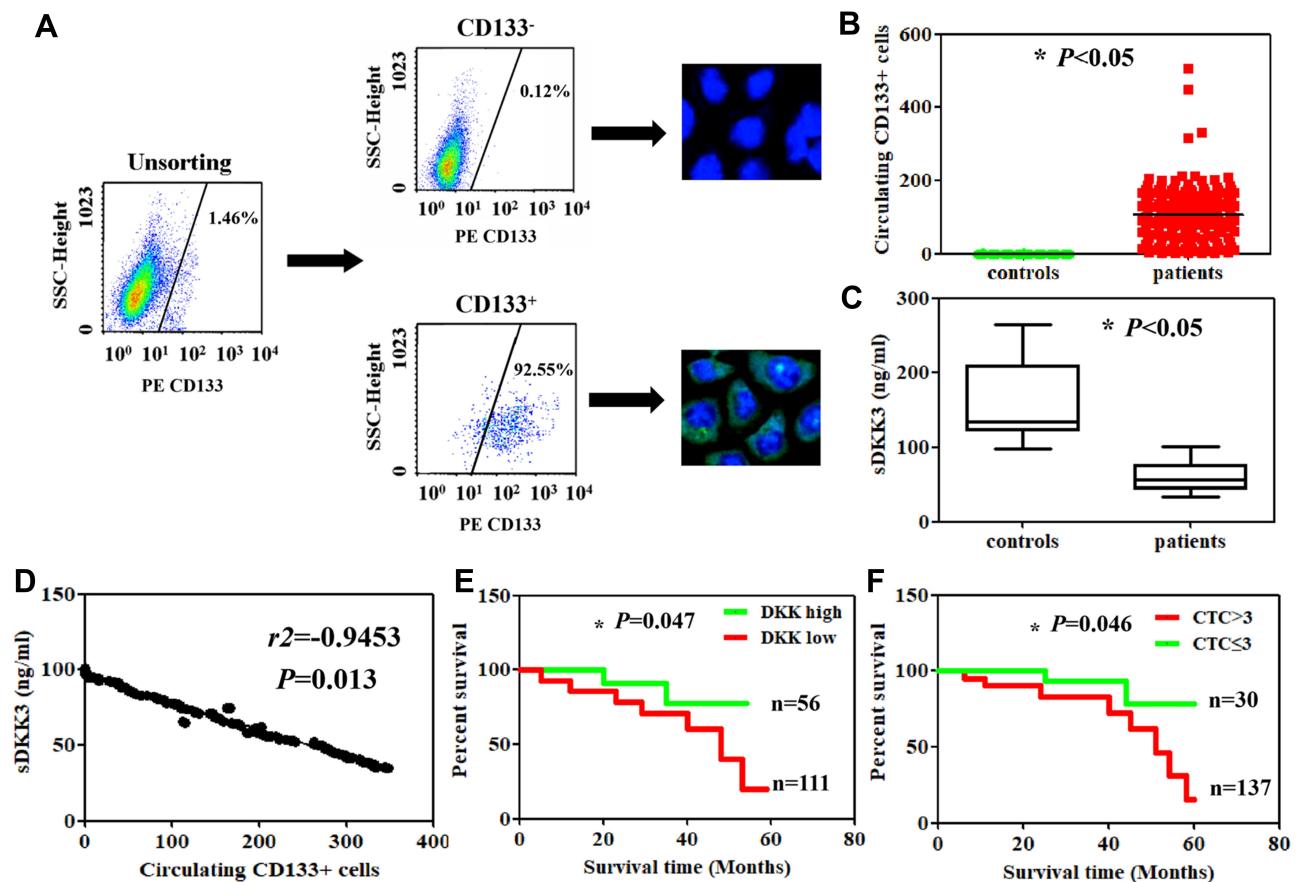


Figure 2 Circulating CD133⁺ cells and sDKK3 in OV patients' blood samples. (A) Distribution of CD133⁺ and CD133⁻ cells was detected by flow cytometry. IF was used to identify the cells. (B) The number of circulating CD133⁺ cells was determined using FCM in patients and healthy controls (n=3, $P < 0.05$, vs control group). (C) Serum levels of DKK3 in patients and healthy controls were evaluated by ELISA (n=3, $P < 0.05$, vs control group). (D) Correlation between sDKK3 and circulating CD133⁺ cell levels was evaluated using the Spearman correlation test ($r^2 = -0.9453$, $P = 0.013$). (E) Kaplan-Meier survival analysis was performed in the OV patients with high or low sDKK3 levels ($P = 0.047$). (F) Kaplan-Meier survival analysis was performed in the OV patients with different numbers of circulating CD133⁺ cells ($P = 0.046$).

in the two cell types ($P < 0.05$) (Figure 3B). The changes in N-cadherin, Vimentin, Snail, and E-cadherin proteins in OV cells with DKK3 treatment were consistent with their mRNA levels ($P < 0.05$) (Figure 3C). Pan-cancer analysis generated an overview of the mechanism for all DKK family members in OV (Figure 3D).

Table 2 List of Real-Time PCR Primers

Gene-Specific Primer	Sequence (5' to 3')
E-cadherin forward	TCGACACCCGATTCAAAGTGG
E-cadherin reverse	TTCCAGAAACGGAGGCCTGAT
N-cadherin forward	TGAGCCTGAAGCCAACCTTA
N-cadherin reverse	AGGTCCCCTGGAGTTTTCTG
Vimentin forward	GAGAACTTTGCCGTGAAGC
Vimentin reverse	GCTTCCTGTAGGTGGCAATC
Snail forward	CCATGCTCCTCTTGCTCTC
Snail reverse	TACAAAAACCCACGCAGACA
GAPDH forward	AAGAAGGTGGTGAAGCAGGC
GAPDH reverse	GTCAAAGGTGGAGGAGTGGG

Discussion

Low DKK3 expression in the tissue and serum of patients with OV has been confirmed.^{18,19} In this study, we analyzed the mRNA levels of members of the DKK family in OV tissues using TCGA database, and found lower *DKK3* levels in cancer tissues than in normal tissues. These results are consistent with a previous study.¹⁹ Although CTCs are diagnostic and prognostic markers for OV patients,¹⁹ in this study, we found a subgroup of CTCs. We isolated the circulating CD133⁺ cells from OV patients. No CD133⁺ cells were observed in healthy volunteers. So, circulating CD133⁺ cells could be used as a diagnostic and prognostic marker for OV patients. The main finding of this study is the negative correlation between serum DKK3 level and circulating CD133⁺ cells in OV. Both markers can indicate the prognosis of OV patients. The target specificity of antibodies may lead to errors in the detection of plasma proteins.²⁰ Our results show that a combination of the two markers could provide a reliable prognosis for OV patients.

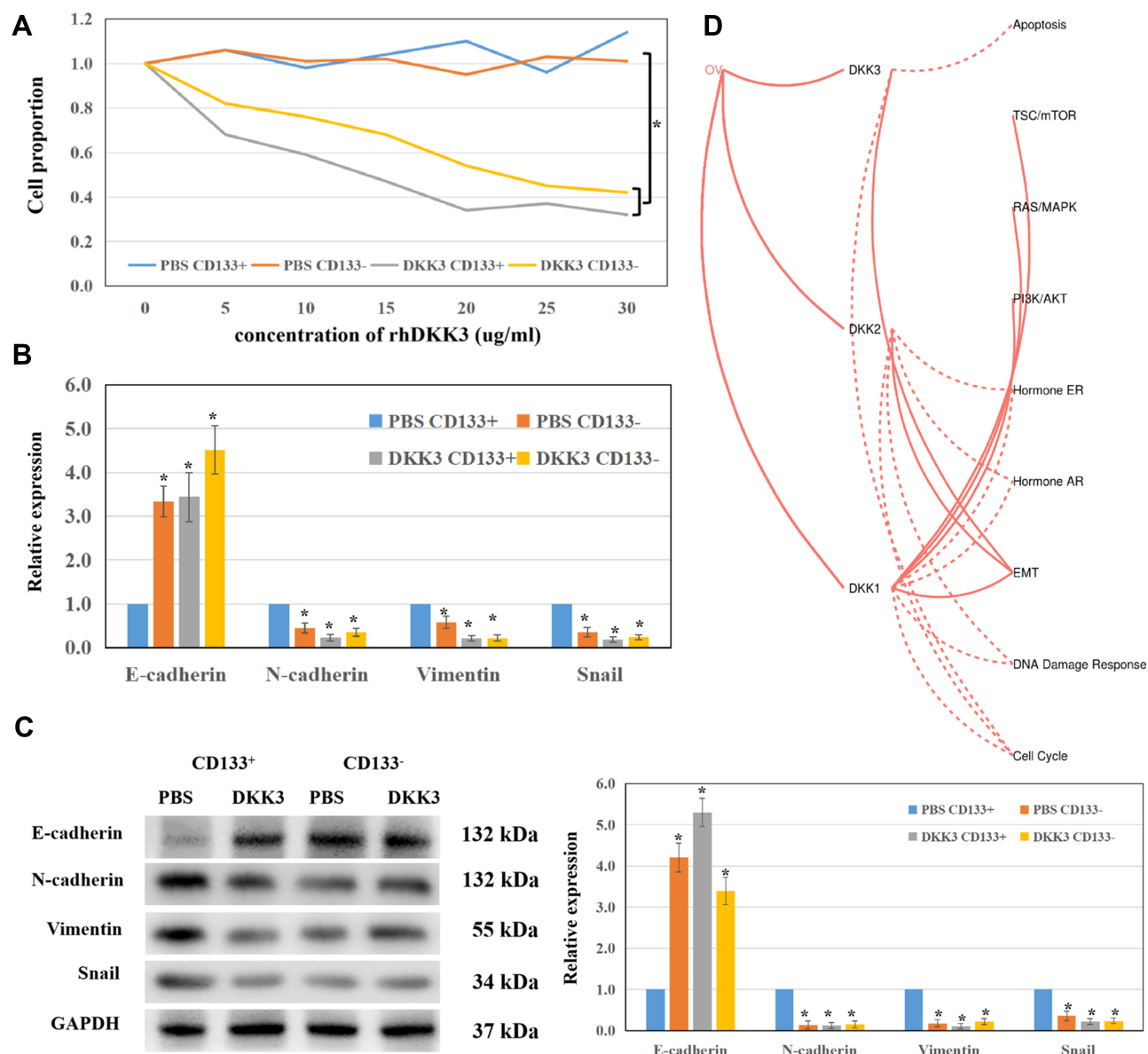


Figure 3 The anti-tumor roles of DKK3 in CD133⁺ cells in vitro. **(A)** The proliferation rate of cells was determined by MTT assay (n=3, *P<0.05, vs PBS CD133⁺ group). **(B)** qPCR was used to check the changes in the EMT pathway (n=3, *P<0.05, vs PBS CD133⁺ group). **(C)** Western blot was used to check the changes in the EMT pathway (n=3, *P<0.05, vs PBS CD133⁺ group). GAPDH was used as the internal control. **(D)** The mechanism of DKK1–4 in OV was analyzed using GEPIA.

In vitro, we confirmed the inhibitory effect of rhDKK3 on CD133⁺ cancer cells. With regard to the mechanism, Western blot and real-time PCR results showed an increase in the epithelial marker E-cadherin and reduced mesenchymal markers (Vimentin, N-cadherin, and Snail) after DKK3 treatment. All the evidence showed that DKK3 reversed the EMT process in CD133⁺ cells. EMT is related to tumor progression and drug resistance.²¹ DKK3 is an inhibitor of the Wnt pathway.¹⁰ Nuclear translocation of β -catenin promotes EMT in OV cells.²² Lu et al²³ showed that curcumin can inhibit the progress of EMT by regulating the WNT signaling pathway. Pan-cancer analysis also showed that

DKK3 could regulate EMT in OV. However, analysis of the results suggests that DKK3 can also regulate the cell cycle and apoptosis via other pathways. This provides us with future research directions. In our previous study, the interaction of DKK3 and CD133 was confirmed.²⁴ This partly explains the stronger inhibitory effect of DKK3 on CD133⁺ cells than on CD133⁻ cells.

In this study, we confirmed the negative correlation between serum DKK3 and circulating CD133⁺ cells in OV patients. Both markers can be used for predicting the outcome of OV patients. In addition, DKK3 played an anti-tumor role in OV cells by reversing EMT.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

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