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ORIGINAL ARTICLE

Correlations of DKK1 with incidence and prognosis of breast cancer

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Summary

Purpose: *To investigate the correlation between Dickkopf-related protein 1 (DKK1) expression and prognosis of breast cancer.*

Methods: *The DKK1 expression in breast cancer cell lines was evaluated via reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. The DKK1 expression pattern in 85 cases of breast cancer was detected using the immunohistochemical method. Besides, the prognostic significance was evaluated via Kaplan-Meier analysis and Cox proportional hazards regression model.*

Results: *DKK1 expression was confirmed in hormone-resistant breast cancer cell lines SK and MDA-MB231. DKK1 expression in breast cancer was associated with cytoplasm/nuclear β-catenin ($p<0.01$). Increased expressions of DKK1 and cytoplasm/nuclear β-catenin in breast cancer indicated adverse outcome of patients. DKK1 was also a bad prognostic factor for patients in early disease stage or without lymph node metastasis.*

Conclusion: *Both DKK1 and β-catenin may be important prognostic factors for breast cancer. DKK1 may be a valuable biomarker for predicting the prognosis of patients in early disease stage or with no lymph node metastasis. β-catenin will possibly serve as a potential therapeutic target for patients with triple-negative breast cancer through further understanding the role of Wnt/β-catenin pathway activation.*

Key words: *breast cancer, correlation, DKK1, prognosis*

Introduction

In the past decade, the gene expression research using DNA microarray has confirmed four common subtypes of breast cancer, a heterogeneous disease. In the gene expression profile analysis, breast cancer is divided into five groups: luminal epithelium/estrogen receptor (ER)-positive A, luminal epithelium/ER-positive B, basal-like, HER2/neu and normal-like carcinoma. The outcome of basal-like carcinoma is poorer compared with luminal tumors. Basal-like carcinoma is characterized by the specific immunophenotype. Breast cancer is negative to ER, progesterone receptor and HER2, and positive to cytokeratin 5/6, 14 and 17, epidermal growth factor receptor (EGFR) and c-kit, which is also related to TP53 mutation. ER-positive tumors include luminal A and B tumors, while ER-negative tumors include basal-like and HER-2-positive tumors. Most basal-like tumors are also known as "triple-negative" (ER-, PR- and HER2-negative) breast cancer [1,2].

Estrogen is necessary for the proliferation of normal mammary epithelial cells [3]. Binding of estrogen to ER activates several gene pathways, including progesterone receptors, and leads to cell proliferation. ER exists in two forms, namely ER-α and ER-β. ER-α is expressed in most breast cancer cells.

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It is reported that there are tendencies of visceral and bone metastases, local recurrence and brain metastasis in patients with triple-negative breast cancer [4,5]. The recurrence and mortality rates of HER2-positive patients have been reduced significantly since the application of trastuzumab and lapatinib, and early recurrence occurs most easily in triple-negative women.

Adjuvant systemic therapy includes chemotherapy, hormone therapy or trastuzumab therapy after the definite surgical resection. Since 1985, the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) has analyzed all available randomized tests once every 5 years, and concluded that the application of adjuvant chemotherapy is related to significant decline in annual recurrence and mortality rates. Adjuvant chemotherapy is more beneficial to young women. Through chemotherapy, the annual recurrence risk and mortality rate are reduced by 37% and 30%, respectively, in women aged <50 years, while they are reduced by 19% and 12%, respectively in women aged 50-69 years [6].

The Wnt/β-catenin pathway is associated with occurrence of breast cancer [7]. Research on mice strongly indicates that the deregulation of β-catenin signal increases the risk of breast cancer through inducing accumulation of stem cells and early progenitor cells [8,9]. Moreover, it has been found that the expression of one gene from MMTV-Wnt1 tumor-initiating cells has prognostic value in basal-like and hormone receptor-negative cancers [10]. According to several studies, cytoplasm and nuclear β-catenin in primary breast cancers, especially basal-like breast cancer, are increased, which is associated with poor prognosis and survival [11-13].

Methods

Ethics approval and consent to participate

The study was approved by the ethics committee of Dongying People's Hospital and written informed consents were signed by the patients and/or their guardians.

Main reagents

Glutamine was purchased from Invitrogen, Carlsbad, CA, USA, TRIzol reagent was purschased from Invitrogen, Carlsbad, USA, miRNeasy micro column was purchased from miRNAeasy Mini Kit, Qiagen, Hilden, Germany, miRNeasy mini column was purchased from Qiagen, Eppendorf Mastercycler 22331 polymerase chain reaction (PCR) instrument was purchased from Eppendorf, Hamburg, Germany, DKK1 and β-catenin were purchased from Abcam, Cambridge, MA, USA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA and FUJIFILM LAS-3000 system was purchased from Fuji Film, Tokyo, Japan.

Cell lines and data sources

Human breast cancer cell lines MDA-MB-231, MCF7, BT-474, SK-BR-3 and Hela were kept in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine under 10% CO_2 at 37°C. The clinical samples of breast cancer were obtained from Dongying People's Hospital. 136 triple-negative [estrogen receptor (ER)-negative, progesterone receptor (PR)-negative and HER-2-negative] patients were continuously selected from 1203 operable breast cancer patients. They were followed up regularly, and clinical results were obtained from 85 patients and updated most recently in June 2015. The median follow-up time was 60 months. All samples were coded anonymously according to local ethical code, and written informed consent was obtained.

Reverse transcription (RT)-PCR

Ribonucleic acid (RNA) extraction and quantitative RT (qRT)-PCR were performed as previously mentioned. The total RNA was isolated from formalin-fixed paraffin-embedded specimens [14]. Micro RNA (miRNA) was extracted from tissues or cells using TRIzol reagent, treated and enriched using the miRNeasy micro column. Briefly, the tissue samples were treated with TRIzol and chloroform, and the mixture was centrifuged at 12000 g and 4°C for 15 min. 100% ethanol in a 1.5-time volume was added into the aqueous layer, and the mixture was treated using the miRNeasy mini column according to recommendations of the manufacturer. Primer sequences of DKK1: forward: AACGCTATCAAGAACCTGC, reverse: GATGACCGGAGACAAACA, target fragment of 460 bp. Primer sequences of GAPDH: forward: GGGAGCCAAAAGGGTCATCATCTC, reverse: CCATGCCAGTGAGCTTCCCGTTC, target fragment of 353 bp. Amplification was performed using the Eppendorf Mastercycler 22331 PCR instrument.

Western blotting

After the cell lysate was centrifuged at 10000 rpm for 10 min, the clear supernatant was taken. Detection was performed using the DKK1 and β-catenin antibodies according to standard regimens. The transfer quality was evaluated via Western blotting of GAPDH antibody, followed by color development using enhanced chemiluminescence (ECL) reagent and image capturing using the FUJIFILM LAS-3000 system.

Immunohistochemistry

Immunohistochemical staining of DKK1 and β-catenin was performed using the avidin-biotin-peroxidase method. Briefly, tissue sections were dewaxed in xylene, rehydrated with gradient ethanol, and boiled in 0.01 mol/L sodium citrate buffer (pH 6.0) in a microwave oven for 10 min for antigen retrieval. After the activity of endogenous peroxidase was blocked with 0.3% hydrogen peroxide, and the non-specific protein binding was blocked with 1.5% normal goat serum, sections

were incubated with antibodies (DKK1 antibody diluted at 1:50 and β-catenin antibody diluted at 1:1000) in a humidifying chamber at 4°C overnight. Then, sections were incubated with the biotinylated goat anti-mouse IgG for 30 min, followed by detection using the LSAB system.

Evaluation of immunohistochemical variables

Results of immunohistochemical staining were evaluated independently by two pathologists who were uninformed of the characteristics of patients. The DKK1 staining was positive in more than 50% tumor cells, in which the staining was dark brown and completely covered the cytoplasm. No obvious staining in tumor cells indicated negative result [14]. In the immunoreactive evaluation of β-catenin protein, cytoplasm/nuclear staining was considered to be positive in more than 10% cells [15].

Statistics

The follow-up time was defined as the time from the operation to the last observation, or from the operation to recurrence/death. The recurrence-free survival time was defined as the time from the initial operation to recurrence or June 2015, and the overall survival time was defined as the time from the initial operation to postoperative recurrence/death or June 2015. The recurrence-free survival and overall survival rates were deduced via the Kaplan-Meier method, and the difference in survival curve was compared using the log-rank test. The two-sided Pearson x^2 test was used for comparison between DKK1-positive and DKK1-negative tumors in the same cluster. $P<0.05$ suggested that the difference was significant.

Results

DKK1 expression in breast cancer cell lines

The DKK1 messenger RNA (mRNA) levels in four different kinds of breast cancer cell lines were compared with that in normal Hela cell line. The DKK1 mRNA expression levels in MCF-7, SK and Hela cell lines were relatively lower than those in

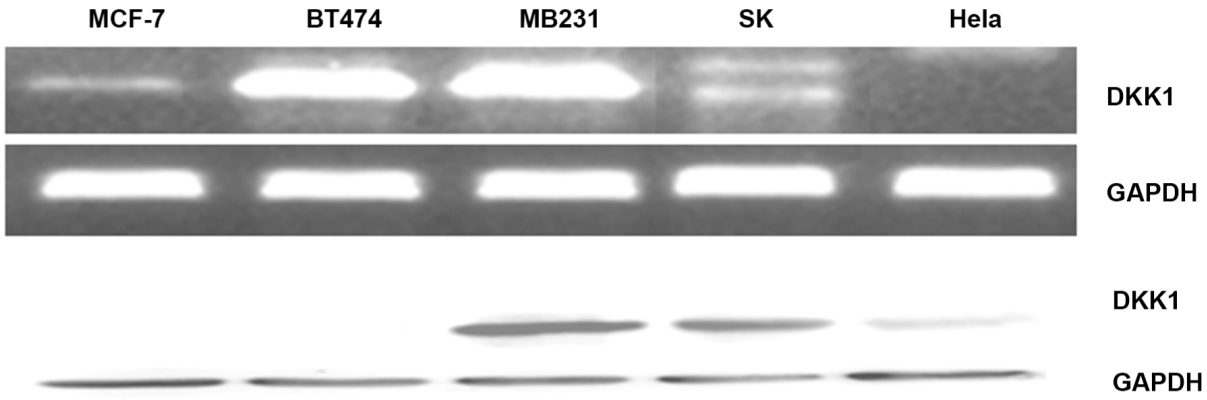

Figure 1. DKK1 mRNA expression and protein expression in breast cancer cell lines.

Table 1. Characteristics of 85 patients with breast cancer

Characteristics	*n*	*%*
Type		
Invasive breast cancer	60	70.59
Others	25	29.41
Lymph node status		
0	50	58.82
≥1	35	41.18
Tumor size, cm		
≤2	32	37.65
>2	53	62.35
Tumor stage		
I-II	65	76.47
III	20	23.53
Total	85	100

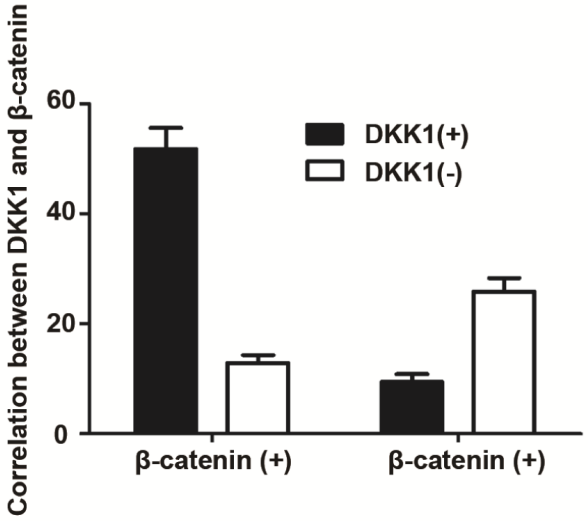

Figure 2. Correlation between DKK1 and β-catenin expressions ($p<0.01$).

BT474 and MDA-MB231 cells. The DKK1 protein expression in these cells was further studied via Western blotting. High DKK1 protein expression was confirmed in the hormone-resistant breast cancer SK and MDA-MB231 cell lines. Interestingly, the DKK1 expression levels were extremely low in hormone-sensitive MCF-7 and BT474 cells (Figure 1).

Characteristics of patients

The correlation between DKK1 and β-catenin expression level in clinical samples of breast cancer was studied via immunohistochemical staining. All tumor samples were invasive breast cancer. Fifty cases had radical mastectomy, 29 local mastectomy, and the remaining 6 cases had breast-conserving operation. After operation, 72 patients underwent adjuvant systemic therapy according to the recognized practice guidelines. During follow-up, 64 (75.29%) patients had no recurrence or metastasis, 32 (37.65%) patients had recurrence or metastasis, and 17 (20.00%) patients died of breast cancer (Table 1).

Immunostaining of DKK1 and β-catenin

DKK1 staining was mainly observed in the cytoplasm of tumor cells. DKK1 was positively

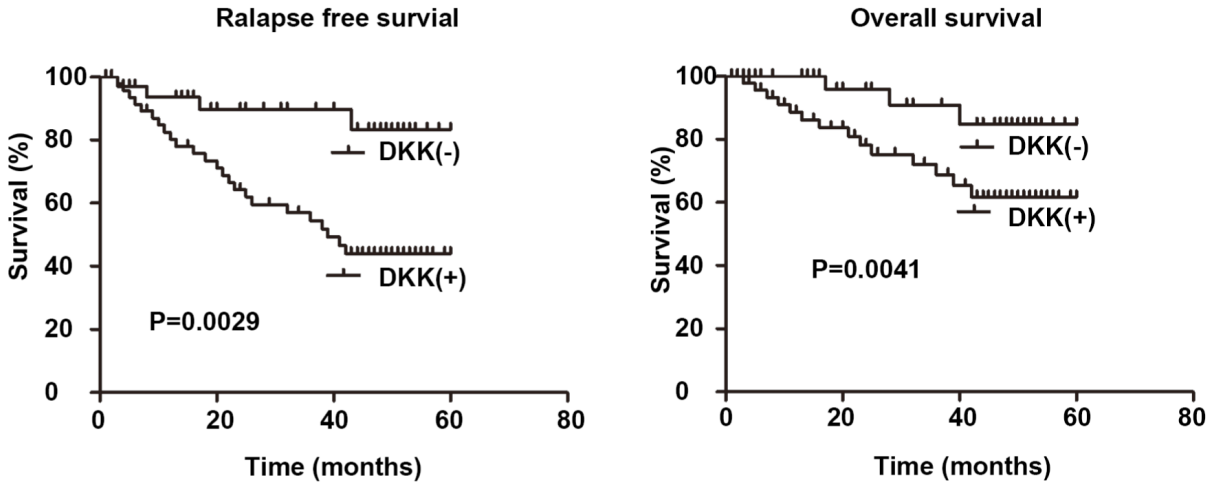

Figure 3. Survival based on DKK1 protein expression.

Table 2. Univariate analyses of prognostic factors for patients with breast cancer

	Recurrence-free survival rate		*Overall survival rate*	
	RR (95% CI)	*p*	*RR (95% CI)*	*p*
Pathological type	0.785 (0.237-1.812)	0.534	0.732 (0.312-1.721)	0.365
Lymph node status	2.321 (1.212-4.532)	0.031	1.923 (0.623-5.211)	0.156
Tumor size	1.672 (0.812-3.621)	0.225	1.432 (0.601-4.221)	0.512
Tumor stage	3.615 (1.821-7.234)	0.001	3.432 (1.345-8.901)	0.015
DKK1	4.912 (1.921-12.654)	0.001	7.201 (1.654-32.011)	0.011
β-catenin	3.125 (1.312-8.012)	0.018	5.623 (1.342-25.213)	0.031

RR: relative risk

Table 3. Multivariate analyses of prognostic factors and adjuvant treatment for patients with breast cancer

	Recurrence-free survival		*Overall survival*	
	RR (95% CI)	*p*	*RR (95% CI)*	*p*
Lymph node status	1.212 (0.437-2.812)	0.834	-	-
Tumor staging	2.625 (0.832-6.534)	0.061	2.422 (1.125-7.911)	0.045
DKK1	3.814 (1.025-8.623)	0.033	3.812 (0.689-17.698)	0.101
β-catenin	1.823 (0.735-5.123)	0.267	3.632 (0.896-17.332)	0.178

RR: relative risk

Table 4. Prognostic value of DKK1 in stage II tumor and lymph node status subgroup

		Overall survival			*Recurrence-free survival*		
		0	*1*	*p*	*0*	*1*	*p*
Stage II tumor	DKK1+	19	3	0.013	19	3	0.013
	DKK1-	20	8				
Lymph node status	DKK1+	22	2	0.023	22	2	0.002
	DKK1-	20	6		14	2	

Table 5. Correlations of DKK1 expression with other clinicopathological parameters

Characteristics	*DKK1+*	*DKK1-*	*p*
Type			
Invasive breast cancer	37	26	0.273
Others	15	7	
Lymph node status			
0	26	24	0.038
≥1	26	9	
Tumor size, cm			
≤2	22	11	0.408
>2	30	22	
Tumor stage			
I-II=0	33	29	0.014
III=1	19	4	

expressed in 52 out of 85 patients, while it was negative in 33. In terms of positioning of β-catenin via immunostaining, cytoplasm/nuclear staining of β-catenin was observed in 55 out of 85 patients with breast cancer, and there was no staining in the remaining 30 patients. Moreover, there was a significant correlation between DKK1 and β-catenin expressions ($p<0.01$) (Figure 2).

Correlations of DKK1 with clinical outcomes of breast cancer patients

There were significant differences in recurrence-free survival and overall survival rates between DKK1-positive and DKK1-negative patients. The recurrence-free survival and overall survival rates of DKK1-negative patients were significantly higher than those of DKK1-positive patients ($p<0.01$), indicating that the DKK1 expression has a significant correlation with survival rate, and DKK1-negative patients have better prognosis (Figure 3).

Univariate and multivariate analyses of prognostic factors and adjuvant therapy for patients with breast cancer

According to the results of correlation analyses between tumor characteristics and outcomes of patients, lymph node status, tumor staging, and DKK1 and β-catenin expressions were significantly associated with recurrence-free survival, while no significant prognostic value was found in other factors listed in Table 2. In terms of overall survival, it was found that tumor staging, DKK1 and β-catenin had significant prognostic value, but such a correlation was not found in other factors (Table 2). Multivariate analyses proved that DKK1 was an independent risk factor for recurrence-free survival and tumor staging (Table 3). The prognostic value of DKK1 in different tumor stages or lymph node status subgroups was further assessed and showed that the recurrence-free survival and overall survival rates of patients with stage II tumor and DKK1-negative tumor were obviously higher than those in patients with DKK1-positive tumor (Table 4).

Correlations of DKK1 expression with other clinicopathological parameters

The correlations of DKK1 expression with tumor characteristic parameters are shown in Table 5. It was found that the DKK1 expression was related to lymph node status and tumor stage. No significant correlations were observed in other tumor parameters.

Discussion

In this study, the DKK1 protein expression in the breast cancer hormone-resistant cell line MDA-MB-231 was reported. Although the DKK1 mRNA expression was detected in the receptor-positive or HER2-positive breast cancer cells in this study, the DKK1 protein expression was not found in these cells. The possible reason is that most of the DKK1 has been secreted from these cells, so DKK1 cannot be detected in cells. Forget et al. reported positive expression of DKK1 in the supernatant of MCF-7 cells detected via ELISA [16].

It was found that the prognosis of patients with positive expressions of DKK1 and β-catenin was poor. At the same time, recurrence-free survival and overall survival of patients with stage II tumors and DKK1-negative tumors were significantly

longer than that of patients with DKK1-positive tumors (recurrence-free survival, p=0.013, overall survival, p=0.013;recurrence-free survival, p=0.002, overall survival, p=0.023, respectively). Therefore, combining the DKK1 expression status with the tumor stage may be helpful to predict the prognosis of patients with triple-negative breast cancer. DKK1 and Wnt/β-catenin are regulated by progesterone in normal endometrial stromal cells. Forget et al. [16] also observed DKK1 in two kinds of hormone-independent prostate cancer cell lines (DU45 and PC3) and hormone receptor-negative breast tumor, but not in the hormone-dependent tumor (LNCaP), which are consistent with the results in this study. Up-regulation of DKK1 was related to the accumulation of cytoplasm/nuclear β-catenin in triple-negative breast cancer in this study, which might indicate the activation of pathway actually.

During the regulation of breast development and tumorigenesis, β-catenin regulates breast stem cells and Wnt/β-catenin signal transduction pathway to play an important role through its correlation with E-cadherin in cell adhesion. Lin and colleagues reported that the nuclear staining of β-catenin is related to poor prognosis of breast cancer patients [11]. However, such a correlation failed to be found in several other studies [17,18]. One possible reason is that β-catenin was not positioned as a molecular subtype in these studies. According to previous reports of Khramtsov et al., Wnt/β-catenin pathway is enriched in basal-like breast cancer. There were significant limitations in our study, including small dataset, few patients in stage I, no verification in independent cases, and classification score of DKK1 and β-catenin in positive and negative breast cancers via immunohistochemistry. Despite these defects, the results of this study supported the idea that the Wnt/β-catenin signaling pathway is activated in triple-negative breast cancer.

Although triple-negative breast cancer accounts only for 15-20% of all breast cancers, it is a severe challenge in clinical practice due to inability to give endocrine therapy and lack of targeted drugs. Currently, treatment strategies include a lot of chemotherapy drugs. Triple-negative breast cancer has a significantly higher response rate to chemotherapy than luminal A or B. However, both disease-free survival and overall survival rates of triple-negative breast cancer are much shorter. The data in this study indicate that β-catenin may be a potential therapeutic target for this subtype of breast cancer. The effect of Wnt pathway inhibitors has been detected in colorectal cancer [19,20], and it is necessary to further study this compound, so as to explore its potential therapeutic effect on triple-negative breast cancer *in vitro* and *in vivo*.

In conclusion, the accumulation of DKK1 and cytoplasm/nuclear β-catenin in patients with triple-negative cancer predicts adverse outcome. DKK1 may be a valuable biomarker for predicting the prognosis of patients in early disease stage or without lymph node metastasis. The potential mechanism between the increased DKK1 expression and activation of Wnt/β-catenin pathway in triple-negative cancer remains unknown. β-catenin may become a potential therapeutic target for triple-negative breast cancer through further understanding the role of Wnt/β-catenin pathway activation.

Conflict of interests

The authors declare no conflict of interests.

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