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Original Article

DDX51 gene promotes proliferation by activating Wnt/β-catenin signaling in breast cancer

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Abstract: Breast cancer was a malignant tumor seriously threatening the life of women in the world. But the prognosis of breast cancer patients was not so satisfactory due to the limited effective therapeutics. The heterogeneity decided that more than one gene or one signaling pathway was responsible for the initiation or progression of breast cancer. DDX51 gene was a member of RNA helicases family in charge of regulation of RNA metabolism. And DDX51 gene was shown to promote proliferation in NSCLC. But we firstly reported the abundant expression of DDX51 gene in both the breast cancer tissues and cell lines in this study. And DDX51 expression was shown to be associated with TNM stage and prognosis in breast cancer patients. When DDX51 was successfully knocked down, either proliferation or DNA synthesis of MCF-7 cells was inhibited. But the ability of migration and invasion of MCF-7 cells was not affected by DDX51 gene. Furthermore, DDX51 knockdown was accompanied by inhibition of Wnt/β-catenin signaling because expression of critical members such as β-catenin, cyclin D1, TCF/LEF, and DKK1 were all affected. Therefore, this study proved that DDX51 gene promoted proliferation in MCF-7 cells by regulating Wnt/β-catenin signaling pathway and showed clinical significance in breast cancer. This study provides us a new promising hope for treatment of patients with breast cancer.

Keywords: DDX51, breast cancer, MCF-7, Wnt/β-catenin signaling

Introduction

DEAD-box family (DDX) consists of a group of RNA helicases with the activity to hydrolyte ATP and to unwind RNA [1]. The major physiological role of this family was to regulate RNA metabolism including ribosome assembly, RNA splicing, and pre-rRNA processing [1-3]. But in recent years, DDX genes were reported to be involved in tumorigenesis of multiple cancers. For example, up-regulated DDX2A was correlated with metastasis of non-small cell lung cancer (NSCLC) [4]. DDX5 contributed to cell proliferation in breast cancer and lung cancer [4]. DDX46 promoted proliferation and inhibited apoptosis in esophageal squamous cell carcinoma cells [4]. But DDX51 was a new gene in DDX family and was composed of 666 amino acids. Besides its proliferation-promoting role in NSCLC, the methylation status of DDX51 was used to differentiate B- and T-acute lymphoblastic leukemia patients [5, 6]. Also it was responsible for the maturation of 3-end of 28S rRNA [7]. But little information of DDX51 gene in breast cancer was seen before.

Breast cancer was one of the most three leading causes of death in women. But the dominant therapies for breast cancer are still the traditional methods consisted of surgery, and radio-chemotherapy [8-10]. Although the antibody based immunotherapy improved the quality of patients with breast cancer, the prognosis was unsatisfactory, if not disappointed [11-14]. According to the reports by WHO in 2017, about 252,710 new women would be diagnosed as breast cancer in the USA. What's worse is that there would be about 40,610 new deaths in the coming year [15]. Both the incidence rate and death rate of patients with breast cancer

Table 1. Primers designed for QPCR of DDX51 gene and GAPDH

Gene	Primers (5-3)		Product length
DDX51	Forward	CCCTGGTTACGGGACAGAAG	197 bp
	Reverse	GTCAATCATCCGGTCAGCCT	
GAPDH	Forward	GACCACAGTCCATGCCATCA	153 bp
	Reverse	CCGTTCAGCTCAGGGATGAC	

Table 2. Three designed fragments of shRNA targeting DDX51 gene

Fragment symbol	Target sequence (5-3)
shDDX51-1	GCAGCTCCGCTTCCTGATTAT
shDDX51-2	GGAAGATCCAGCTGCTCATCA
shDDX51-3	GCTGCTCTTCTCAGCTACTCT

increased steadily in the nearest three years. Therefore, it is in no time to develop new therapies to treat women with breast cancer.

Breast cancer is known to be characterized by heterogeneity. Unsatisfactory prognosis of breast cancer may be partly attributed to heterogeneity [16]. But canonical Wnt/β-catenin pathway played a very important role in breast cancer. Wang et al reported that prodigiosin could suppress proliferation of breast cancer cells by inhibiting Wnt/β-catenin signaling [17]. Niclosamide was shown to sensitize triple-negative breast cancer cells to ionizing radiation through blocking of Wnt/β-catenin signaling [18]. DDX3 was indeed one subunit of casein kinase1 of Wnt/β-catenin signaling [19].

Wnt/β-catenin signaling has been involved in the whole process in nearly all kinds of cancers. In the canonical Wnt pathway, β-catenin is necessary for the activation of Wnt signaling. GSK-3β is a glycogen synthase kinase which could phosphorylate β-catenin and cause degradation of β-catenin. Upon binding of Wnt to its receptor, signaling protein dishevelled suppresses the destruction complex containing GSK-3β and leads to accumulation of β-catenin followed by translocation to the nucleus. Then β-catenin binds to TCF/LEF factors and activates downstream genes [20-22].

In present study, we explored the function of DDX51 in breast cancer by determining its effect on MCF-7 cells and our data suggested that DDX51 was an oncogene in breast cancer and regulated the Wnt/β-catenin signaling in breast cancer. This study will provide a new clue for therapy of breast cancer patients and push forward the process to cure breast cancer.

Materials and methods

Cell lines and breast tumor tissues

Human breast cancer cell lines were bought from Cell Bank Type Culture Collection of Chinese Academy of Sciences (CBTCCCAS, China) and maintained in DMEM (Gibco, USA) containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C.

57 patients samples diagnosed with breast cancer were collected from Affiliated Cancer Hospital of Zhengzhou University. Written informed consent was obtained from each individual subject. And all experiments were approved by the Ethics Committee of Affiliated Cancer Hospital of Zhengzhou University.

Quantitative real-time polymerase chain reaction (QPCR)

Total RNAs from breast cancer tissues or cells were extracted using Trizol agent followed by treatment with RQ1RNase-free DNase. cDNA was prepared from 1 μg of total RNA by SuperScript III g (Invitrogen, USA) according to the manufacturer's instructions. Then SYBR Green RT-PCR Kit (Takara, Japan) was used to detect the relative mRNA level of DDX51 on ABI 7500 real-time PCR system (Life Technologies, USA). GAPDH was the internal control. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. Primers used for QPCR were shown in **Table 1**.

Preparation of lentivirus expressing shRNA targeting DDX51 (lenti-shDDX51) and cell infection

Three shRNA fragments targeting DDX51 gene were designed and displayed in **Table 2**. Then each sequence was synthesized and subcloned into expression vector pLKO.1-GFP-shRNA. After confirming by DNA sequencing, the expression vector containing shDDX51 was used to prepare the lentivirus particle Lenti-shDDX51.

Then MCF-7 cells were seeded into a 6 well plate at 5×10^5 cells/well, infected with Lenti-

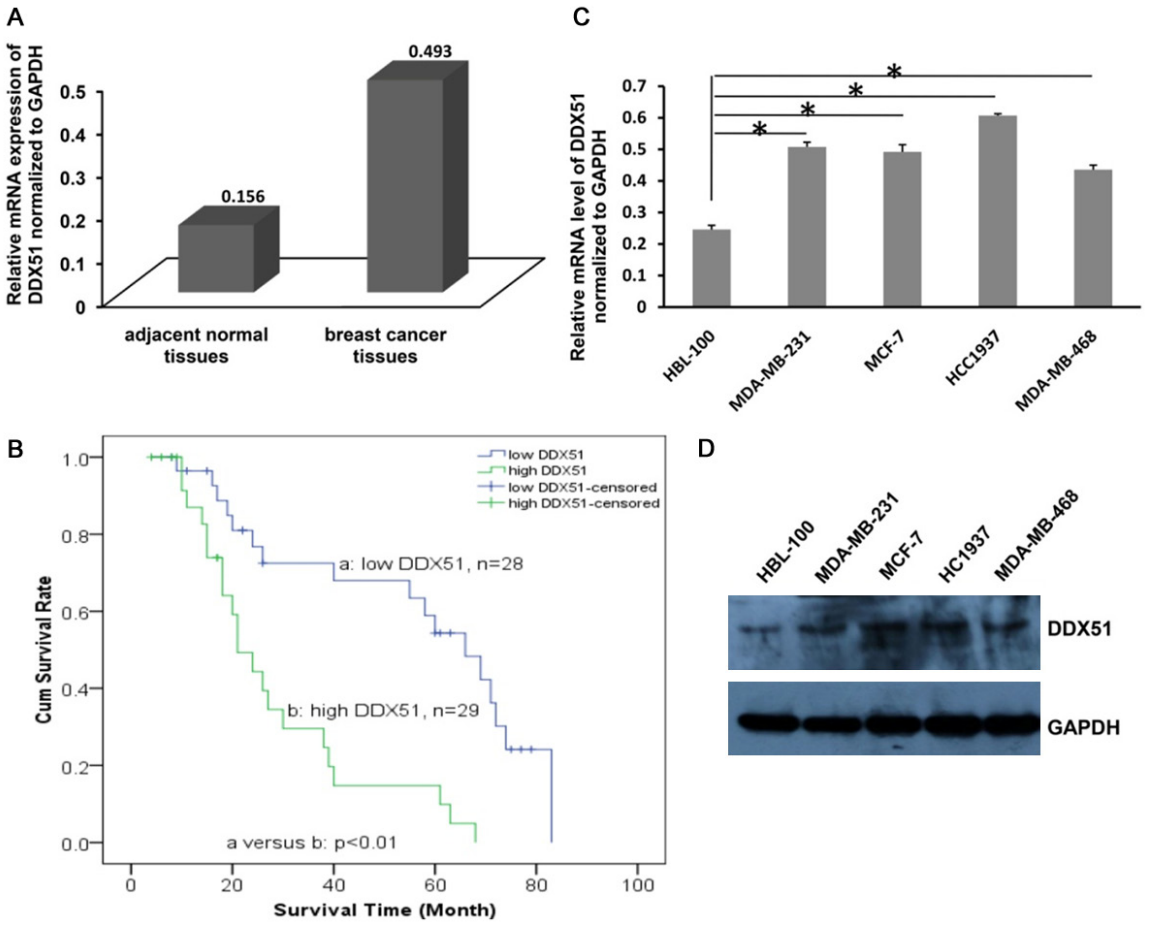

Figure 1. DDX51 was overexpressed in breast cancer and was associated with 5-year survival rate. A. DDX51 was overexpressed in 57 breast cancer tissues compared to adjacent normal control. B. DDX51 level was associated with 5-year survival rate in patients with breast cancer. C and D. DDX51 was expressed much higher in breast cancer cell lines than in normal HBL-100 cells at mRNA level and protein level. *P<0.05 was considered statistically significant.

shDDX51 at 20MOI after culture for 24 h. Then cells were cultured before further treatment.

Cell proliferation assay (MTT assay)

MCF-7 cells were seeded into 96-well plates at 3000 cells/well in 200 μl DMEM (Gibco, USA). 20 μl of MTT solution (Sigma, 5 mg/ml) was added daily, and plates were incubated for another 4 h at 5th day before removing the supernatant. After that, 100 μl of dimethyl sulfoxide (Sigma, USA) was added to dissolve the crystals. Absorbance values (A) at 490 nm were measured and recorded.

BrdU incorporation assay

The infected cells were seeded to a 96-well plate at 2000 cells/well. And 10 μl of 1× bromodeoxyuridine (BrdU) was added and cells were cultured for another 24 h or 72 h. After that, about 100 μl fixing solution was added for 30 min before washing with wash buffer. Then cells were incubated with 50 μl 1× BrdU antibody for 1.5 h before 50 μl Goat anti-mouse IgG antibody and 50 μl TMB substrate solution were added. At last, stop solution was added and incubated for 30 min and absorbance value (A450 nm) was determined on a microplate reader.

Cell migration assay (wound scratch assay)

Cells were seeded into a 6 well plate at 2×10^5. After culture for 24 h, a scratch was created using a 10 μl tip, and the debris were removed with PBS gently. Then cells were cultured for

Table 3. Correlation of DDX51 gene expression with clinicopathological features in breast cancer

Factors	Folder change of DDX51		*P* value
	≥3	<3	
Age			0.708
≥50	20	18	
<50	9	10	
TNM classification			0.005
I/II	11	21	
III/IV	18	7	
Lymph node metastasis			0.91
Positive	17	16	
Negative	12	12	

another 24 h in serum-free DMEM. At 2 h and 24 h, the width in five randomly selected fields were observed and recorded. The migration rate was calculated with the equation below:

Migration rate = $(S_{2\,h}-S_{24\,h})/S_{2\,h}$ ×100%

$S_{2\,h}$ stands for the width of the scratch at 2 h while $S_{24\,h}$ stands for the width at 24 h.

Cell invasion assay (transwell assay)

About 1×10^4 infected cells were placed into an upper chamber (8.0 μm pore size, Millipore, USA) pre-coated with 1 mg/ml Matrigel (BD Biosciences, China). The upper chamber was located in a 24-well plate. And 600 μl new medium with 20% FBS was used as chemoattractant in the lower chamber. Then the cells were cultured for 48 h in the CO_2 incubator at 37°C. The upper cells were removed and the penetrated cells were stained with dye solution (0.1% crystal violet and 20% methanol). Then the cells in five randomly selected fields under a microscope (Olympus, Japan) were calculated.

Western blot analysis

Cells were cultured and collected for total protein extraction using protein extraction Kit (Sigma, USA). Then the concentration of total protein was determined by with BCA Protein Assay Kit (Generay, China). 15 μg total protein were separated on a SDS-PAGE gel, electrotransferred to PVDF membranes, and incubated for 12 h with primary mouse monoclonal antibodies against beta-catenin, Myc (Santa Cruz, USA) and LEF1, TCF4, cyclin D1, GAPDH (Abcam, USA). Membranes were then treated with horseradish peroxidase conjugated secondary antibody for overnight at 4°C. Immunoreactivity was detected with an enhanced chemiluminescence kit (Amersham Biosciences, USA).

Statistical analysis

All experiments were repeated for three times and all data were shown as mean ± standard deviation and analyzed by Student's t-test for statistical difference with SPSS16.0. The association of DDX51 gene expression with clinicopathological characteristics was analyzed by chi-square test. The relationship of DDX51 gene expression with survival rate was analyzed by the Kaplan-Meier method and compared by the log-rank test. *P* value <0.05 was considered significance between two groups.

Results

DDX51 expression was associated with prognosis in breast cancer patients

Compared with the normal adjacent breast tissues, DDX51 gene expression was increased in cancer tissues. As shown in **Figure 1A**, the mean level of DDX51 mRNA in breast cancer tissues was about 3.6 fold higher than that in the adjacent tissues. The clinicopathological analysis as in **Table 3** revealed that DDX51 expression was associated with tumor stage (TNM I/II versus III/IV, P<0.05). Moreover, we found that the 5-year survival rate of breast cancer patients with high DDX51 expression was only 13.8% which was significantly worse than those (46.4%) with low DDX51 expression (P<0.01) (**Figure 1B**). In addition, we found that DDX51 gene was expressed in abundance in typical breast cancer cell lines as in **Figure 1C** and **1D**. Therefore, it was implicated that DDX51 gene was important in breast cancer.

DDX51 gene was successfully knocked down in MCF-7 cells

To study the function of DDX51 gene in breast cancer, we reduced the expression of DDX51 in MCF-7 cells by lentivirus mediated RNAi (LentishDDX51) technology. As shown in **Figure 2A** and **2B**, the infection efficiency of lentivirus on

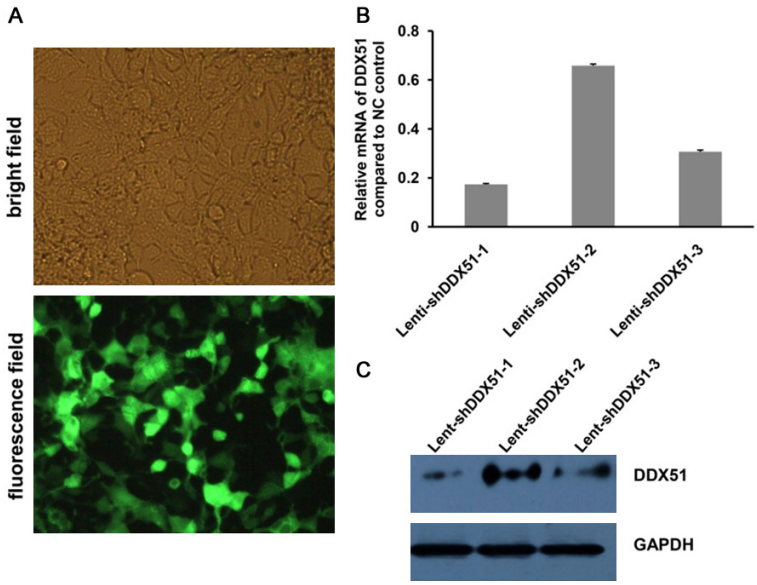

Figure 2. DDX51 was knocked down in MCF-7 cells by lentivirus mediated shRNA. A. The infection efficiency of lenti-shDDX51 particle on MCF-7 cells. B and C. DDX51 expression was decreased at both mRNA level and protein level.

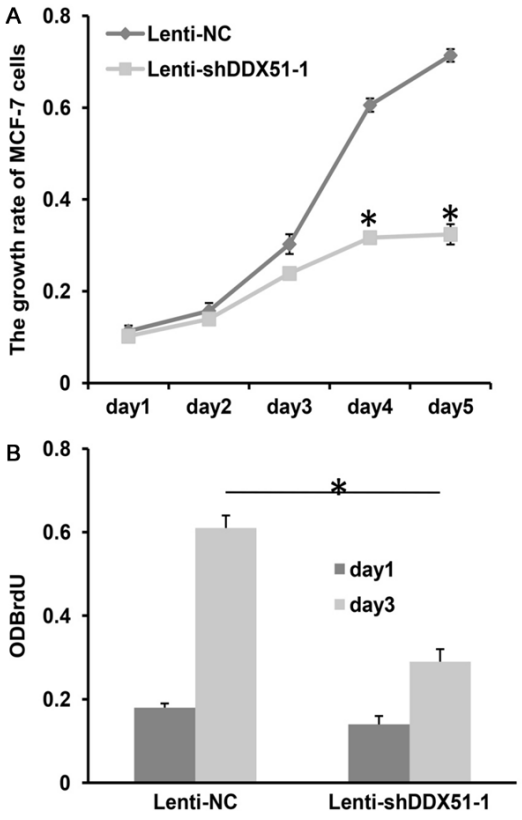

Figure 3. Decreased DDX51 suppressed cell proliferation and DNA synthesis. A. DDX51 knockdown inhibited cell growth of MCF-7 cells in MTT assay. B. DNA synthesis was suppressed after DDX51 knockdown in BrdU incorporate assay. *P<0.05 was considered statistically significant.

MCF-7 cells was above 90%. Then it was not surprising that the mRNA level of DDX51 in test group was decreased by 80% (**Figure 2C**). Furthermore, we confirmed the knockdown efficiency of DDX51 at protein level (**Figure 2D**). Conclusively, DDX51 gene was successfully knocked down in MCF-7 cells.

DDX51 knockdown inhibited proliferation and DNA synthesis of MCF-7 cells

We designed the MTT assay to detect the growth and proliferation of MCF-7 cells. As in **Figure 3A**, the cells number in Lenti-shDDX51-1 group decreased to about 45.4% of that in control group at 120 h post infection. Then in the BrdU incorporation assay, we found that DNA content in Lenti-shDDX51-1 group was only about 47.5% compared to the control (**Figure 3B**). So knockdown of DDX51 greatly inhibited proliferation and DNA synthesis in MCF-7 cells.

Migration and invasion of MCF-7 cells was not affected by DDX51

We investigated the effect of DDX51 knockdown on invasion of MCF-7 cells in transwell assay with matrigel on the membrane. Unexpectedly, we demonstrated that DDX51 had little effects on invasion ability of MCF-7 cells (**Figure 4A** and **4C**). Then the wound healing assay further confirmed the results (**Figure 4B** and **4D**).

Canonical Wnt/β-catenin signaling was blocked after DDX51 knockdown

In **Figure 5A**, we found that the level of β-catenin, cyclin D1, TCF, and Myc was drastically decreased after DDX51 was knocked down in MCF-7 cells. But DKK1 was increased reversely. When LGK-974 was used to treat MCF-7 cells in parallel, we got the same results. Meanwhile, the proliferation rate of MCF-7 cells was more or less the same in LGK-974 group as in Lenti-shDDX51-1 group. But there was no additive effect between LGK-974 and Lenti-shDDX51-1 (**Figure 5B**). Based on these results, we con-

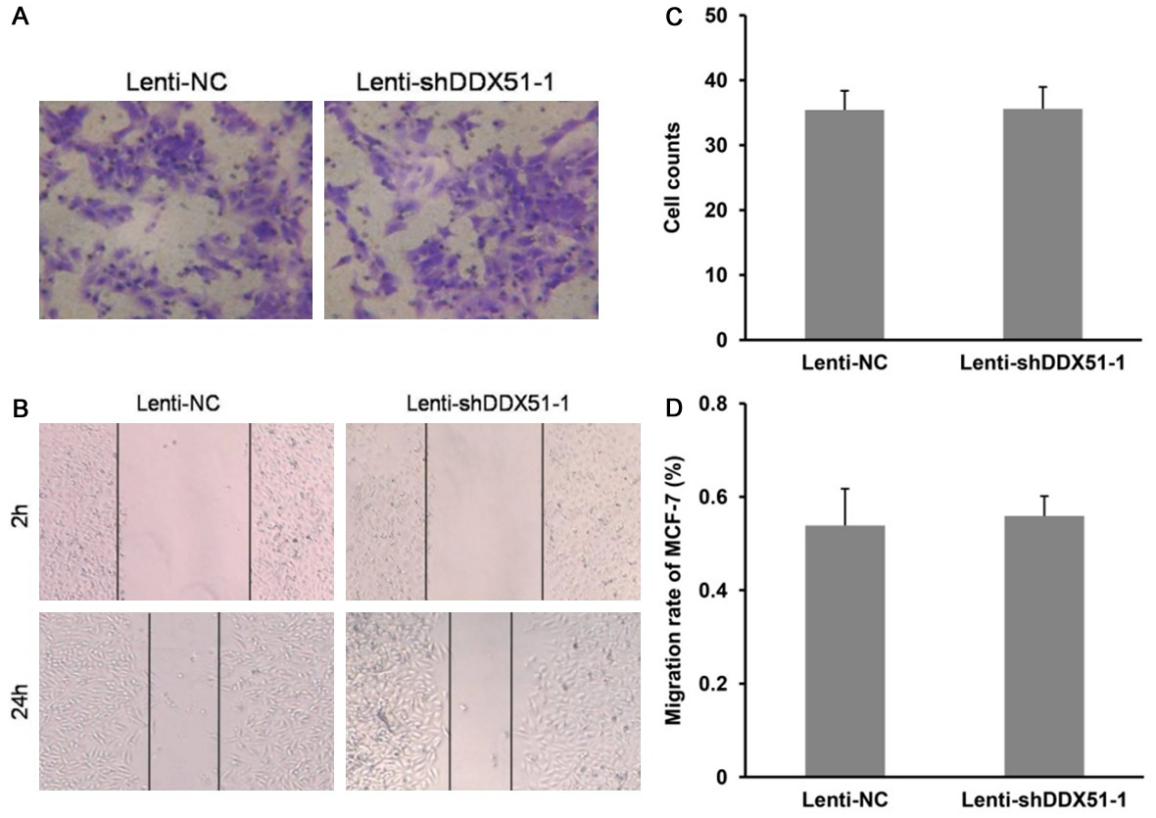

Figure 4. The ability of migration and invasion of MCF-7 cells was not affected by DDX51. A and C. DDX51 knockdown didn't affect cell invasion of MCF-7 in transwell assay. B and D. DDX51 knockdown didn't affect cell migration of MCF-7 cells in wound scratch assay.

cluded that DDX51 knockdown blocked activation of Wnt/β-catenin signaling in MCF-7 cells.

Discussion

Tumor is characterized by heterogeneity and multiple tumor behaviors could be attributed to heterogeneity such as tumor recurrence, drug resistance, tumor cell metastasis and so on [23-25]. Next generation sequencing has demonstrated that several dozens or even several hundreds of mutations occurred in each kind of tumor genome. Transformation of normal cell to tumor cell may be a dose-dependent effect of gene mutation. But only several genes or even one single gene were shown to be the driver gene. And the driver gene was different due to tumor heterogeneity [26]. Breast cancer was also a heterogeneous tumor [27]. Here, we proved DDX51 gene as a new driver gene in breast cancer. As shown in the above text, DDX51 gene was essential to the proliferation and DNA synthesis of MCF-7 cells. There are about ten hallmarks in tumor, one of which is unlimited cell proliferation. As in the above text, DDX51 gene contributed greatly to the proliferation of breast cancer cells. Breast cancer was reported as a malignant tumor with potential aggressive feature [28]. And potent invasive ability was also another hallmark of tumor cells. However, the migration or the invasion ability of MCF-7 cells was not affected by DDX51 gene. Based on these data, we could conclude that DDX51 gene prompted cell proliferation but not invasion in breast cancer.

Cell proliferation is a critical behavior for either normal development or tumor growth. And more than one signaling pathway was involved in the regulation of cell proliferation. For example, Wnt/β-catenin signaling, PI3K/mTOR pathway, NFκB signal, and some other signaling pathway all regulate cell proliferation and cross with each other to form a signal network [29-31]. In this study, we found that DDX51 knockdown in MCF-7 cells significantly reduced the

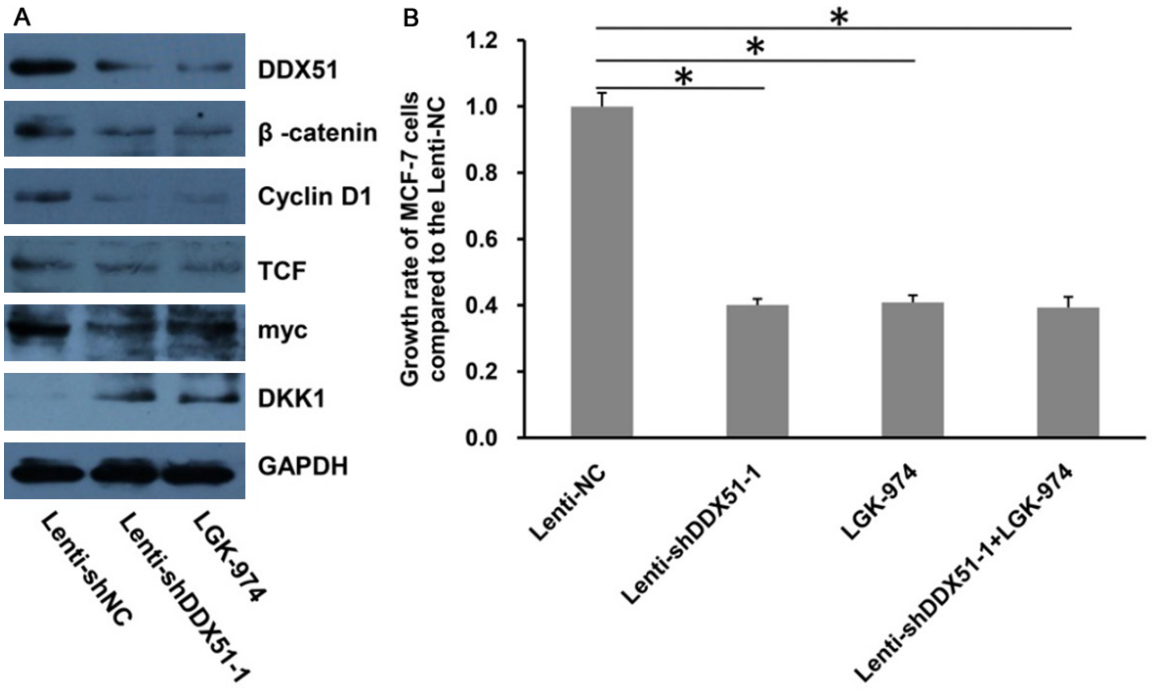

Figure 5. DDX51 promoted cell proliferation through regulating Wnt/β-catenin signaling pathway. A. DDX51 knockdown regulated expression of critical molecules in Wnt/β-catenin signaling pathway. B. There was no significant additive effect between DDX51 knockdown and Wnt inhibitor LGK-974 on cell proliferation of MCF-7 cells. *P<0.05 was considered statistically significant.

expression of β-catenin, cyclin D1, TCF, LEF molecules but enhanced the expression of DKK1. Accumulation of β-catenin was a typical and critical event during the activation of β-catenin-dependent Wnt signaling pathway [29]. Meanwhile, DKK1 was generally an inhibitor molecule and down-regulated in tumor cells. So it was easily to understand that DDX51 gene promoted proliferation in breast cancer cells MCF-7 by activation of typical Wnt/β-catenin signaling pathway. In addition, Myc gene was also shown to be down-regulated after DDX51 knockdown. The level of Myc gene was increased in a series of tumors and was responsible for cancer cell proliferation [32]. Myc gene was shown as a downstream target of Wnt/β-catenin signaling in many kinds of tumors [33]. So it was conceived that activation of Wnt/β-catenin signaling increased the level of Myc gene followed by cell proliferation in breast cancer. And this conclusion was further supported by the results obtained from the small molecule LGK974 which was a specific inhibitor of typical Wnt signaling. As shown in the above, no additive effects were discovered between LGK974 agent and DDX51 knockdown.

DDX51 gene was shown to promote cell proliferation in NSCLC [5]. But no further information about its clinical role in cancer was reported previously. In this study, we demonstrated that DDX51 gene level in breast cancer tissues was greatly increased compared to the adjacent normal tissues. And higher DDX51 gene expression could be used to predict the prognosis of patients with breast cancer. Also, the relationship between DDX51 gene expression and tumor stage was disclosed here. All these data come to a conclusion for the first time that DDX51 gene is a very important factor in clinic for breast cancer patients. DDX51 gene may be a valuable indicator for monitor of therapy of patients with breast cancer. However, these clinical data was based on a relative small number of breast cancer patients. And verification of DDX51 gene on a large cohort of clinical samples is essential before translational application. Also, the in vivo antitumor effects of DDX51 gene would further reflect its value in breast cancer and these will be an important part of our next job.

In summary, this study proved the clinical significance of DDX51 gene in breast cancer for

the first time. And DDXA51 gene was shown to promote proliferation as well as DNA synthesis through activation of canonical Wnt signaling in breast cancer cells MCF-7. This study will provide a new valuable clue for the therapy and prognosis of breast cancer patients.

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Disclosure of conflict of interest

None.

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