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Downregulated CDKN1C/p57^{kip2} drives tumorigenesis and associates with poor overall survival in breast cancer

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ABSTRACT

CDKN1C, also known as p57^{kip2}, is considered to be a potential tumor suppressor implicated in several kinds of human cancers. However, the current knowledge of CDKN1C in breast cancer remains obscure. In the present study, we demonstrated that CDKN1C was dramatically downregulated in breast cancer compared with normal tissues by using real-time quantitative polymerase chain reaction, western blot and two public data portals: The Cancer Genome Atlas (TCGA) and Oncomine datasets. Moreover, the expression of CDKN1C was correlated with age and tumor size in the TCGA cohort containing 708 cases of breast cancer. Low expression of CDKN1C was significantly associated with poor overall survival (OS) in the TCGA cohort and validated cohort composed of 1402 patients. Multivariate Cox regression analysis indicated that CDKN1C was an independent prognostic factor for worse OS (HR = 1.78, 95% CI: 1.09–2.89, $p = 0.020$). Furthermore, gene set enrichment analysis (GSEA) revealed that CDKN1C was significantly correlated with gene signatures involving DNA repair, cell cycle, glycolysis, adipogenesis, and two critical signaling pathways mTORC1 and PI3K/Akt/mTOR. In conclusion, our data suggested an essential role of CDKN1C in the tumorgenesis of breast cancer. Targeting CDKN1C may be a promising strategy for anticancer therapeutics.

1. Introduction

Breast cancer is the most common malignancy and also the principle cause of cancer-related mortality among women worldwide. According to the global cancer statistics, an estimated 1.7 million women were diagnosed as new cases of breast cancer and more than 0.5 million patients died from this disease in 2012 [1]. As a heterogeneous carcinoma, the treatment effects and clinical outcomes are obviously different among patients with different molecular subtypes [2]. On the other hand, several molecular features such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER-2) have been used in the current management of breast cancer [3]. However, it is limited to choose treatment strategies and predict survival outcomes relying on these factors. Identification of new biomarkers is urgent for better prediction of therapeutic efficiency and clinical outcomes in breast cancer patients.

CDKN1C, also known as p57^{Kip2}, is the most recently found cyclin-dependent kinase inhibitor. The gene encoding CDKN1C is localized at chromosome 11p15.5 [4]. CDKN1C belongs to the kinase inhibitor protein/CDK-interacting protein (Kip/Cip) family, which includes three members namely CDKN1A/p21^{Cip1}, CDKN1B/p27^{Kip1} and CDKN1C/p57^{Kip2} [5]. All Kip/Cip family members share similar domains for the regulation of cell cycle progression by binding to and inhibiting a broad range of cyclin-dependent kinases (CDKs) [5,6]. Consistent with the role of CDK inhibitor, the expression of CDKN1C was upregulated in G0/G1 phase and downregulated during the transition of G1 to S phase [7]. Previously, CDKN1C was identified as a tumor suppressor with reduced expression level in different cancers including hepatocellular carcinoma [8], colorectal cancer [9], and ovarian cancer [10]. Accordingly, upregulation of CDKN1C resulted in the inhibition of hallmarks involving cell growth, differentiation, cell death, and angiogenesis in malignancies [7]. It is noteworthy that low expression of CDKN1C was associated with worse clinical outcomes

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for cancer patients, a clue which shows the potentiality of CDKN1C serving as a prognostic indicator [7,11]. However, the biological role of CDKN1C in breast cancer has not been well documented and remains largely unknown.

In the present study, we first investigated the expression of CDKN1C based on publicly available transcriptional data and collected breast cancer tissues. Clinical significance of CDKN1C was then determined in large breast cancer cohorts. Finally, gene set enrichment analysis (GSEA) was performed to explore potential functions and molecular mechanisms of CDKN1C mediated the progression of breast cancer.

2. Materials and methods

2.1. Patients and tissue samples of breast cancer

Tumor and adjacent normal tissues used for real-time quantitative polymerase chain reaction (RT-qPCR) and western blot were collected from breast cancer patients treated by primary surgery between 2014 and 2016 at the First Affiliated Hospital of Chongqing Medical University. All specimens were immediately snap-frozen in liquid nitrogen and stored at −80 °C until used for RNA isolation and protein extraction. This study was approval by the Institutional Ethics Committees of the First Affiliated Hospital of Chongqing Medical University. All patients received an explanation of the study aims and signed informed consent.

For The Cancer Genome Atlas (TCGA) cohort, clinical and gene expression data was downloaded from Cancer Genomics Browser of University of California Santa Cruz (UCSC) (https://genome-cancer.ucsc.edu/), version: 2015-02-24. We included breast cancer patients only with complete RNAseq data and fully clinical information including tumor size, lymph node status, TNM stage, ER, PR, HER-2, and overall survival (OS) data (version nature-2012). In total, 708 cases of breast cancer patients were included in the present study. The RNAseq data of all 117 normal samples were used for the comparison of mRNA expression difference between tumor and normal tissues.

2.2. RNA isolation and RT-qPCR

Total RNA was isolated using TRIzol reagent (Life Technologics Inc. USA) following manufacture introduction. RNA concentration was determined by spectrophometry with a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). A total of 1 μg RNA was subjected to reverse transcription to cDNA by Reverse Transcription Kit (Promega Inc. USA). RT-qPCR was carried out by ABI 7500 Real-Time PCR System (Applied Biosystems) using Maxima SYBR Green/ROX qPCR Master Mix (MBI Fermentas, St. Leon-Rot, Germany). Primer pairs used were as follows: CDKN1C, forward primer: 5′-TGA ACG CCG AGG ACC AGA AC-3′, reverse primer: 5′-TGC ACC GTC TCG CGG TAG A-3'; β-actin, forward primer: 5′-CCT GTG GCA TCC ACG AAA CT-3′, reverse primer: 5′-GAA GCA TTT GCG GTG GAC GAT-3'. Thermal cycling conditions were 95 °C for 30 s, followed by 5 s at 95 °C, 1 min at 60 °C for 40 cycles. Relative quantification mRNA expression levels of CDKN1C were standardized to β-actin.

2.3. Protein extraction and western blot

Total proteins were extracted using RIPA lysis buffer (Pierce, Thermo Scientifc, Cramlington, UK). The concentration of proteins was determined by BCA protein assay kit (Pierce Biotechnology Inc., Rockford, USA). A total of 40 μg proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). After blocked by 5% non-fat milk 1 h at RT, membranes were incubated with primary antibody CDKN1C (Cell Signaling Technology, Inc., Danvers, MA, CST #2557) and β-actin (Abcam Inc., Cambridge, MA, ab8226) overnight at 4 °C. After washed 3 times with TBST, the membranes were incubated with HRP-conjugated secondary antibody at 1:5000 dilutions for 1 h at RT. Protein bands were visualized by an ECL chemiluminescence systemand short exposure of the membranes to X-ray films (Kodak, Japan). Densitometric analysis was performed with Image Pro-Plus software. Relative protein expression levels of CDKN1C were normalized to β-actin.

2.4. Bioinformatics analyses

Oncomine (http://www.oncomine.org), an online microarray database containing publicly available microarray data for multiple human cancers, was utilized to examine the mRNA expression difference between breast cancer and normal tissues. The thresholds were restricted as follows: p-value = 1E-4; fold change = 2; gene rank = 10%; data type: mRNA. Fold change, t-test value, p-value and sample size were abstracted from comparisons with statistical significance.

Kaplan-Meier plotter database (http://kmplot.com/analysis/) [12] was used to determine the prognostic value of CDKN1C in breast cancer. Patients were divided into high and low expression groups by auto selected cut-off value of CDKN1C mRNA expression level. The desired probe ID (213348_at) was entered into the database to obtain Kaplan-Meier Plot.

2.5. Gene set enrichment analysis (GSEA)

GSEA was performed using the GSEA software 3.0 from the Broad Insitute as previously described [13]. The gene microarray data (GSE1456) was downloaded from GEO database (https://www.ncbi.nlm.nih.gov/geo/). The gene set of "h.all.v6.1.symbols.gmt", which summarize and represent specific well-defined biological states or processes, was downloaded from Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb/index.jsp). Normalized enrichment score (NES) was acquired by analyzing with permutations for 1000 times. A gene set is considered as significantly enriched when a normal p-value < 0.05 and false discovery rate (FDR) < 0.25.

2.6. Statistical analysis

Statistical analyses were performed by SPSS version 22.0 software and Graphpad 5.0. The expression comparison of CDKN1C between tumor and normal samples was estimated using student's t-test. The associations of CDKN1C with clinicopathological features were assessed by chi-square test or Fisher's exact test when needed. The OS curve and its significance were determined by Kaplan-Meier method and log-rank test, respectively. Univariate and multivariate analyses of prognosis were carried out by Cox proportional hazard regression model. All p values were two-tailed and considered statistically significant when less than 0.05.

3. Results

3.1. The expression pattern of CDKN1C in breast cancer

We first investigated the mRNA expression difference of CDKN1C using the mRNA HiSeq expression data from TCGA. As shown in Fig. 1A, the mRNA expression level of CDKN1C was decreased in breast cancer compared with normal tissues. Within Oncomine database, CDKN1C mRNA was significantly downregulated in different subtypes of breast cancer tissues compared

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