Int J Clin Exp Pathol 2016;9(6):6476-6482
www.ijcep.com /ISSN:1936-2625/IJCEP0024816

Original Article

CDK4 amplification is associated with distant metastasis and poor clinical outcome in breast cancer

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Received January 24, 2016; Accepted April 22, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: Amplification of the cyclin-dependent kinase 4 (*CDK4*), located at 12q13-q14, has been reported in various human tumors including breast cancer. The aim of this study was to assess *CDK4* gene amplification in invasive ductal carcinoma (IDC) with clinical implications. Quantitative PCR (qPCR) was performed on formalin-fixed and paraffin-embedded (FFPE) tumor samples for gene amplification detection. The clinical histopathological characteristics and prognostic significance were analyzed. Of the 157 IDC patients, CDK4 gene amplification was found in 18 (11.5%). CDK4 amplification was associated with distant metastasis (after initial surgery) (P=0.009). In survival analysis, it was also associated with disease-free survival (DFS, P=0.026) and overall survival (OS, P=0.020). With multivariate analysis showed that CDK4 amplification was found to be associated with DFS (amplification vs non-amplification, hazard ratio, 4.456; 95% confidence interval, 1.383-14.353; P=0.012). With respect to treatment regimen, this is also true for DFS (P=0.014 for chemotherapy and P=0.010 for radiotherapy). Patients with CDK4 amplification is associated with distant metastasis after initial surgery and favors poor clinical outcome.

Keywords: Breast cancer, *CDK4*, gene amplification, metastasis, prognosis

Introduction

Based on the 2014 World Health Organization (WHO) report, breast cancer is the second most life-threatening tumor after lung cancer for women in China [1]. Many of the tumor-suppressor genes and oncogenes altered in cancer are known to affect cell cycle regulation. Disruption of the cell cycle machinery might enhance genomic instability, contribute to uncontrolled cell growth, and lead to the development of cancer [2, 3]. The *CDK4* gene encodes a 33-kD protein that plays an important role in the regulation of the G1-S transition of the cell cycle. CDK4, in complex with cyclin D1, can phosphorylate Rb, inactivating the protein and releasing negative control, thus allowing the G1/S transition to precede [2, 4]. CDK4 activity is deregulated in many human tumors [3, 5].

Numerous genes were found to be abnormal in breast cancer with different biological significance [6]. Gene amplification is an important and common mechanism for oncogene overexpression in many tumors. Amplification and consequent overexpression of the *CDK4* gene, located in the 12q13-q14 region, have been found in various cancers including different types of sarcomas and glioblastomas [7-9]. Previous studies show that *CDK4* amplification and overexpression were associated with higher breast tumor cell proliferation rate, but no clinical characteristicwere reported [10]. The *CDK4* amplification has been detected with fluorescent differential PCR and next generation sequence (NGS) at different rate with no clinical outcome implications [6, 10]. Furthermore, a significant proportion of breast cancer exhibits dysregulation of the CDK4/cyclin D1/Rb inter-

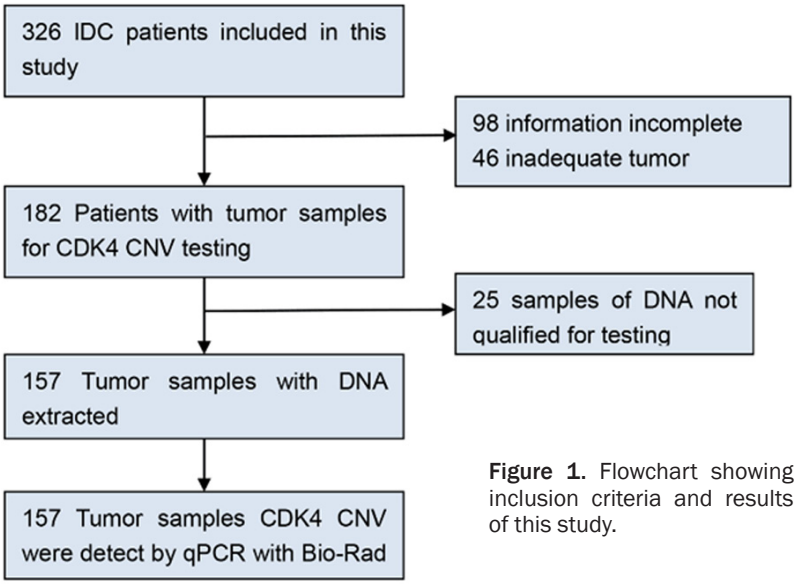

Figure 1. Flowchart showing inclusion criteria and results of this study.

action, potentially indicating a role for targeted therapies. Certain CDK4/6 inhibitors such as PD0332991 (palbociclib), LEE011 (ribociclib), and LY2835219 (abemaciclib) were already in clinical trial [11-13]. In this study we aimed to assess whether there are patients with amplification of *CDK4* in IDC. Further we also analyzed the clinical significance for *CDK4* amplification in breast cancer patients, e.g., tumor size, invasion, and metastasis, as well as prognostic values and therapy responses.

In our study, we find that *CDK4* amplification is associated with distant metastasis after initial surgery and, our data indicate that the *CDK4* amplification favors poor clinical outcome and might be a biomarker for breast cancer target therapy.

Materials and methods

Patients and sample preparation

The samples were human breast neoplasm tissue removed during surgery. Patients' anonymity was preserved in all of the cases. Approval for the study was granted by the Ethics Committee of West China Hospital (No. 2013-191). We analyzed formalin-fixed and paraffin-embedded (FFPE) sample from 157 patients with breast cancer who underwent breast mastectomy between 2010 and 2012 at West China Hospital (**Figure 1**). Surgical specimens were obtained before systemic treatment, and paraffin embedding was performed within the framework of diagnostic procedures. Disease-free survival (DFS) and overall survival (OS) were defined as the time between the initial surgery and local or distant metastatic relapse, and the time between surgery and death, respectively.

DNA isolation and quantitative polymerase chain reaction

Tumor areas (at least 1 cm^2) from 4.0 μm-thick unstained FFPE sections were macrodissected. DNA was isolated from two 4 μm-thick tissue sections using a QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quantitation was performed using a nanodrop 2000 (Thermo, Waltham, MA, USA). Finally, DNA purity was confirmed by measuring the A260/280 absorbance ratios. Good-quality DNA was indicated with the ratio A260 nm/A280 nm=1.70-1.95. Reactions were carried out using a Bio-Rad CFX96 system (Applied Biosystems, Hercules, CA, USA). Each gene was measured in triplicate and normalized relative to a set of two reference genes (*GAPDH*, *TFRC*) (**Table 1**). The relative quantitation of *CDK4* gene amplification in IDC was calculated by the $2^{-\Delta\Delta CT}$ method using the average copy number in 50 normal breast tissues besides tumor as control samples and reference genes (*GAPDH, TFRC*). The sample was considered positive for *CDK4* gene amplification if the ratio was greater than 2.0, whereas a ratio less than 2.0 indicated that sample was negative [14, 15] (**Table 1**). We confirmed the cut-off value of qPCR through *HER2* in IDC (data unpublished). For detailed quantification method please refers to the previous studies [16, 17].

Statistical analysis

Statistical analyses were conducted using SPSS version 16.0 software (SPSS Inc., Chicago, USA), and a 5% two-tailed significance level was considered to be statistically significant. Associations between the prevalence of

Table 1. qPCR primers of the CDK4 and reference genes

Gene	GenBank No.	Oligo name	Oligo sequence	Target size
TFRC	NC_000003.12	TFRCF	5'-ACTTCCTCTCTCCCTACGTATC-3'	105 bp
		TFRCR	5'-GCAGTTTCAAGTTCTCCAGTAAAG-3'	
GAPDF	NG_007073.2	GAPDFF	5'-CCTCAAGATCATCAGCAATGCCTC-3'	100 bp
		GAPDHR	5'-GTGGTCATGAGTCCTTCCACGATA-3'	
CDK4	NG_007726.3	CDK4F	5'-GGGTGGGACTCAAGCAATATAC-3'	144 bp
		CDK4R	5'-CCTCACCTCCTTCACACATTAC-3'	

Table 2. Baseline clinical characteristics of the study subjects

	Total No. (n=157)	Disease-free Survival		Overall Survival	
	No. (%)	Log-rank	*P*-Value	Log-rank	*P*-Value
AGE	27-75 (49.1)	0.157	0.692	0.086	0.770
≤50 years	94 (59.9)				
>50 years	63 (40.1)				
GRADING†		3.245	0.072	0.257	0.612
G1-G2	46 (30.5)				
G3	105 (69.5)				
TUMOR SIZE†		1.595	0.207	5.112	0.024*
T0-2	146 (94.2)				
T3-4	9 (5.8)				
NODAL STATUS†		3.800	0.051	4.484	0.034*
N0	69 (44.8)				
N1-N3	85 (55.2)				
CLINICAL STAGE†		3.773	0.052	3.418	0.064
I-II	115 (74.7)				
III-IV	39 (25.3)				
ER STATUS		2.612	0.106	0.484	0.487
ER+	52 (33.3)				
ER-	104 (66.7)				
PR STATUS†		3.729	0.053	0.827	0.363
PR+	61 (39.6)				
PR-	93 (60.4)				
HER2†		6.027	0.049*	1.148	0.563
0-1+	84 (54.2)				
2+	36 (23.2)				
3+	35 (22.6)				

ER=estrogen receptor; PR=progesterone receptor; HER2=human epidermal growth factor receptor 2; †Number differences reflect missing data. *Statistically significant.

CDK4 amplification and clinical parameters were evaluated with a chi-squared test. Univariate survival analysis was conducted using the Kaplan-Meier method, and multivariate survival analysis was carried out using the Cox proportional hazard model.

Results

Baseline clinical characteristics

All of the patients included in this study were female, ranging in age from 27 to 75 years (mean, 49.1 years). The mean DFS was 27.2 months, and the mean OS was 28.3 months. The DFS and OS of the 157 patients are listed in **Table 2** with respect to histopathologic characteristics and prognostic factors, including age, histology grading, tumor size, nodal status, metastasis, and clinical stage, ER, PR and HER2/neu. As expected, HER2/neu (P=0.049) was found to be significantly correlated with DFS. Her2/neu overexpression was found associated with shorter DFS. However, only tumor size (P=0.024) and nodal metastasis status (0.034) were significantly associated with OS. Larger tumor size and a positive node status were found associated with shorter OS (**Table 2**).

Clinical histopathological features of CDK4 amplification in breast cancer

In our study, 18 of 157 patients (11.5%) were detected CDK4 amplification with qPCR. To identify any correlation between the gene amplification status of the *CDK4* and clinical characteristics (**Table 3**), we analyzed the correlation between *CDK4* amplification and clinical features. The patients with *CDK4* amplification were analyzed, respectively, regarding age, histology grading, tumor size, nodal status, clinical stage, ER, PR, HER2/neu status, local recurrence and distant metastasis. In our study, *CDK4* amplification was significantly associated with distant metastasis (after initial surgery) (P=0.009); additionally,

Table 3. Prevalence of *CDK4* amplification in breast tumors stratified according to clinical characteristics

	CDK4 amplification		
	Pb (n=18)	N (n=139)	*P*-value
	No. (%)	No. (%)	
AGE			
≤50 years	13 (72.2)	81 (58.3)	0.256
>50 years	5 (27.8)	58 (41.7)	
GRADING†			
G1-G2	4 (22.2)	42 (31.6)	0.418
G3	14 (77.8)	91 (68.4)	
TUMOR SIZE†			
T0-2	16 (88.9)	130 (94.9)	0.306
T3-4	2 (11.1)	7 (5.1)	
NODAL STATUS†			
N0	7 (41.2)	62 (45.3)	0.750
N1-N3	10 (58.8)	75 (54.7)	
CLINICAL STAGE†			
I-II	10 (58.8)	105 (76.6)	0.111
III-IV	7 (41.2)	32 (23.4)	
ER STATUS			
ER+	4 (22.2)	48 (34.8)	0.288
ER-	14 (77.8)	90 (65.2)	
PR STATUS†			
PR+	7 (41.2)	54 (39.4)	0.889
PR-	10 (58.8)	83 (60.6)	
HER2†			
0-1+	9 (50.0)	75 (54.7)	0.853
2+	4 (22.2)	32 (23.4)	
3+	5 (27.8)	30 (21.9)	
RECURRENCE			
YES	1 (5.6)	3 (2.2)	0.389
NO	17 (94.4)	136 (97.8)	
DISTANT METASTASIS			
YES	5 (27.8)	11 (7.9)	0.009*
NO	13 (72.2)	128 (92.1)	

ER=estrogen receptor; PR=progesterone receptor; HER2=human epidermal growth factor receptor 2; †Number differences reflect missing data. *Statistically significant.

CDK4 amplification primarily occurred in tumors with a high histological grade (**Table 3**).

CDK4 amplification for IDC prognosis

To further reveal the prognostic value of gene amplification for *CDK4* in IDC patients, we evaluated the *CDK4* amplification status with DFS and OS by Kaplan-Meier analysis. In this study,

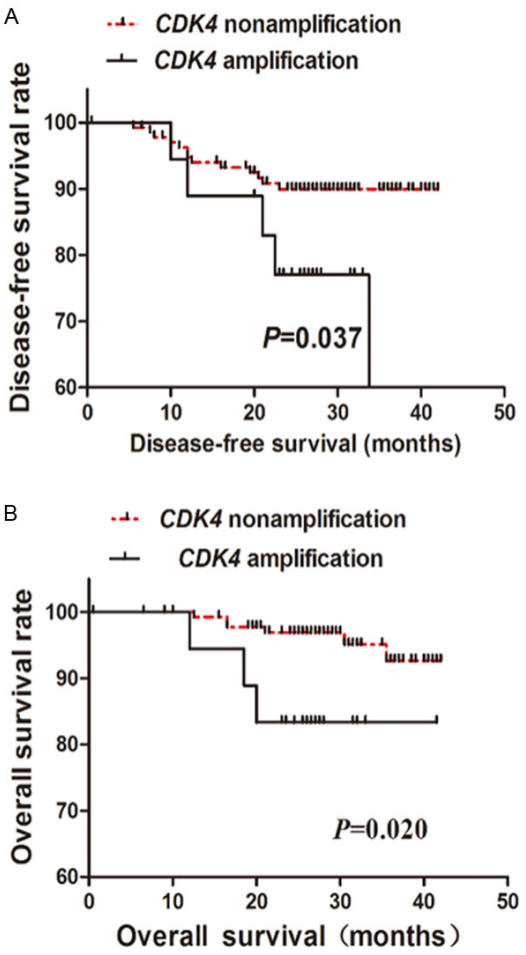

Figure 2. DFS and OS according to *CDK4* gene amplification. Association of *CDK4* gene amplification with prognosis in IDC calculated by the log-rank test and shown by Kaplan-Meier curves. Univariate survival analysis of *CDK4* gene amplification was performed in patients for DFS (A) and OS (B).

we analyzed the *CDK4* amplification group versus nonamplification group for DFS and OS. We found that the patients with *CDK4* amplification had a significantly shorter DFS (P=0.026) and OS (P=0.020) than nonamplification (**Figure 2**).

Furthermore, multivariate analysis showed that CDK4 amplification was found to be associated with DFS (amplification vs nonamplification, hazard ratio, 4.456; 95% confidence interval, 1.383-14.353; P=0.012). But we found that *CDK4* amplification did not associated with OS (**Table 4**). Concerning the treatment regimen, we found that the *CDK4* amplification patients were also significantly correlated with poor DFS, regarding both chemotherapy (P=0.014) and radiotherapy (P=0.010) (**Table 5**).

Table 4. Multivariate Cox Analysis of the histopathologic characteristics and *CDK4* amplification Relationship with the Likelihood of DFS and OS

	DFS	OS
Variable	*P*-value	*P*-value
Age ≤50 years vs >50 years	0.445	0.545
Histologic grade I, II, r	0.283	0.894
PT ≤5 cm vs >5 cm	0.615	0.091
Stage I, II vs III, IV	0.936	0.292
PN 0 VS 1, 2, 3	0.059	0.070
ER protein	0.727	0.974
PR protein	0.277	0.432
HER2 protein	0.316	0.675
co-amplification vs no co-amplification	0.012*	0.100

ER=estrogen receptor; PR=progesterone receptor; HER2=human epidermal growth factor receptor 2. *Statistically significant.

Table 5. Prevalence of *CDK4* amplification and treatment response

		Disease-free Survival	
Treatment	No. (%)	Log-rank	*P*
Chemotherapy	153		
CDK4 amplification	17 (11.1%)	6.089	0.014*
CDK4 nonamplification	136 (88.9%)		
Radiotherapy	52	6.615	0.010*
CDK4 amplification	6 (11.5)		
CDK4 nonamplification	46 (88.5)		
Hormonal therapy	98	1.813	0.178
CDK4 amplification	13 (13.3)		
CDK4 nonamplification	85 (86.7)		

*Statistically significant.

Discussion

The copy number of *CDK4* gene has been determined in a group of 157 invasive ductal carcinoma patients with an average follow-up of 28.3 months and compared with clinical pathological features. The 11.5% (18/157) patients were detected with *CDK4* amplification of IDC in the present study. This subgroup was significantly correlated with a higher possibility distant metastasis (after initial surgery, P=0.009), The *CDK4* amplification was found to be significantly associated with DFS (P=0.026) and OS (P=0.020). Concerning the treatment regimen, we found that the *CDK4* amplification patients were significantly correlated with poor DFS, regarding chemotherapy (P=0.014) and radiotherapy (P=0.010) Thus, *CDK4* amplification can be an independent prognostic indicator.

In our study, the *CDK4* amplification rate was 11.5%, a value similar to that in a previous study (15%) [6, 10]. It was previously shown that *CDK4* amplification and overexpression were associated with high breast tumor cell proliferation, but without any other clinical characteristic [10]. We further investigated the relationship between *CDK4* amplification with the clinical prognosis of breast cancer. Some clinical pathological analyses of *CDK4* amplification in breast cancer with NGS or Fluorescent Differential PCR, but limited to lack of clinical outcomes [6, 10]. We detected *CDK4* gene amplification found that *CDK4* amplification in our present study was significantly associated with shorter DFS and OS. When we analyzed the *CDK4* amplification subgroup with chemotherapy and radiotherapy, we found that the *CDK4* amplification subgroup was significantly correlated with DFS (P=0.014 for chemotherapy and P=0.010 for radiotherapy). However, due to the relatively short follow-up, we could not determine the association between the amplification subgroup and OS regarding the treatment regimens.

Resistant to treatment regiments including chemotherapy, radiotherapy, hormonal therapy and target therapy is a nearly universal, ultimately lethal consequence for breast cancer patients [18-20]. Many theories were used for explanation for drug resistance during treatment, e.g. cancer stem cell theory, EMT theory, special somatic tumor cell mutation [21-23]. Because *CDK4* amplified tumor cells were abnormal in corresponding cell cycle, the patients may response quite different for the treatment regimens. In our study, patients with *CDK4* amplification show poor clinical outcome for both DFS and OS. Interestingly, this is also true with DFS with respect to treatment regimen for chemotherapy (P=0.014) and radiotherapy (P=0.010). Further we wonder how this subgroup of patients' response for the target therapy, e.g. Herceptin treatment for HER2 amplified patients. However, only 9 patients in this study received Herceptin treatment. Only two patients were *CDK4* amplified. One patient was found with distant metastasis (brain) after initial sur-

gery and then dead in two years, but there is no statistically significant data shows the resistance of Herceptin treatment in *CDK4* amplified patients. We also further invested *CDK4* amplification subgroup response to the hormonal therapy, 99 patients received hormonal therapy. 13 (13.3%) patients were *CDK4* amplified. However there are no statistically significant data shows the resistance of hormonal treatment in *CDK4* amplified patients due to limited patients we included in this study. Maybe *CDK4* is a key regulator of cell cycle, so the *CDK4* amplification may be sensitive to the endocrine therapy. Further researches on the treatment response of different treatment on this special subgroup of patients should be carried out. Certain CDK4/6 inhibitors such as PD0332991 (palbociclib), LEE011 (ribociclib), and LY2835-219 (abemaciclib) were already in clinical trial [11, 12]. The CDK4/6 inhibitor (palbociclib, ribociclib and abemaciclib) may be the *CDK4* amplification target medicine [24].

In summary, we detected *CDK4* gene amplification using qPCR, and the results suggest that *CDK4* amplification has considerable prognostic relevance regarding the clinical outcome in breast cancer. *CDK4* amplification can be a novel special subgroup in invasive ductal breast cancer that can be considered predictive of poor clinical outcomes. Regarding treatment regimen analysis, the result of this study indicates that patients with *CDK4* amplification show resistance to chemotherapy, radiotherapy and may be sensitive to hormonal therapy. Special treatment regimens may be required for this special subgroup of patients.

Acknowledgements

This work was funded by grants from the National Natural Science Foundation of China (31000601, 81200461) and Young Investigator Scholar in Sichuan University (2012SCU04A14).

Disclosure of conflict of interest

None.

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