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ORIGINAL ARTICLE

# CKS1BP7, a Pseudogene of CKS1B, is Co-Amplified with IGF1R in Breast Cancers

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**Abstract** Pseudogenes have been reported to exhibit functional roles. Amplification or overexpression of CDC28 protein kinase regulatory subunit 1B (CKS1B) was found in various human cancers. But it was known little about CKS1B pseudogene 7 (CKS1BP7), a pseudogene sharing considerable sequence identity with CKS1B. The aim of this study was to evaluate copy number alterations (CNAs) of CKS1BP7 and address its potential roles in breast cancer. We detected copy numbers of CKS1BP7 and insulin-like growth factor 1 receptor (IGF1R) using quantitative multigene fluorescence in situ hybridization (QM-FISH) technique, compared their status in both invasive carcinoma and ductal carcinoma in situ (DCIS) components within the same tumors, and investigated the associations of CNAs with tumor features and patients outcomes. Amplification of CKS1BP7 (dot-like pattern) was found in 28.8% of all cases, while amplified IGF1R (cluster pattern) was identified in 24.2% of all patients. The two events often co-existed ($p = 0.01$). Within the same tumors, identical CNAs of CKS1BP7 and IGF1R were found in DCIS and invasive carcinoma. Moreover, amplification of both genes was more frequent in aneuploidy tumors and the tumors with high ki67, but wasn't associated with patients' outcome. In summary, CKS1BP7 amplification is a frequent event in breast cancer and often co-occurs with amplified IGF1R, which provides evidence supporting the interactions between CKS1BP7 and IGF1R during mammary carcinogenesis. Our findings suggest that CKS1BP7 as well as IGF1R may serve as potential biomarkers for early detection and predict prognosis in breast cancer.

**Keywords** Breast cancer · CKS1BP7 · IGF1R · Quantitative multi-gene fluorescence in situ hybridization

## Introduction

Breast cancer is a complex disease caused by a combination of genomic alterations in a large number of genes including deletions and amplifications. Copy number alteration (CNA) can alter the ability of the cancer cells to proliferate, survive, and spread in the host and therefore is now recognized as the main events during the initiation and development of cancer and a characteristic of most breast cancers [1, 2]. Therefore, the investigation of CNAs in breast cancer is of high relevance for improving the diagnosis and prognosis of breast cancer patients.

Cytogenetic and molecular studies on breast cancer have identified recurrent genetic alterations including loss on 1p, 4p, 8p, 11q, 13q, 16q, 17p and 22q and gain on 1q, 8q, 11q, 16p,17q, and 20q [3–8], leading to increased knowledge of the genetic basis of breast cancers over the past years. And some of them have become major molecular targets for breast cancer treatment. However it is still largely unknown about the genomic changes of breast cancers and there likely are other

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breast cancer susceptibility genes that are still uncovered. Currently fluorescence in situ hybridization (FISH) is still the rapid, most precise assay to assess gene copy numbers. Using quantitative multi-gene FISH (QM-FISH) procedure, CNAs of multi-genes could be measured in individual cells and analyzed based on the morphology avoiding intra-tumor genomic heterogeneity to the largest extent.

In previous study, we screened the genes involved in breast cancer and found amplification of CDC28 protein kinase regulatory subunit 1B pseudogene 7 (CKS1BP7) in cancer samples. CKS1BP7 belongs to pseudogenes which are found on different chromosomes from their functional counterparts [9], and is mapped to chromosome 8q21, a region of DNA that is frequently amplified in breast cancers. So far we know little about the roles of CKS1BP7. Being cognate gene of CKS1BP7, CDC28 protein kinase regulatory subunit 1B (CKS1B) is also known as cyclin kinase subunit 1 (CKS1). Recent studies have investigated the overexpression of CKS1B in different human cancers, such as breast cancer, esophageal carcinoma, gastric carcinoma, hepatocellular carcinoma and melanoma [10, 11]. Further, amplification or overexpression of CKS1B was found in aggressive disease of multiple myeloma [12] and was associated with lymph node metastasis and poor prognosis in breast cancers [10]. Recently, several studies indicated that the effects of CKS1B on cell proliferation might be related with insulin-like growth factor 1- insulin-like growth factor 1 receptor (IGF1-IGF1R) signaling [13, 14].

A growing body of evidence has been reported that pseudogenes exhibit functional roles with their parent genes and are known to play a key role in the development of cancer and contribute to tumor progression [15]. Taken all the above data together, it is here proposed CKS1BP7 may have a potential role in breast cancer. Meanwhile it is interesting to know if aberrant IGF1R involved in this event. In the present study we detected CNAs of CKS1BP7 and IGF1R in a cohort of 66 breast cancers by QM-FISH assay. Then CKS1BP7 and IGF1R status was compared in invasive carcinoma and ductal carcinoma in situ (DCIS) components within the same tumors and analyzed the associations of CNAs with clinicopathological features and patients outcomes.

## Materials and Methods

### Patients and Tumor Characteristics

We searched the pathology databases of Qilu Hospital, Shandong University, Jinan, China, from January to June 2005 to retrieve cases of non-specified invasive breast carcinoma with concurrent DCIS component. None of the patients received either neoadjuvant chemotherapy or radiation therapy prior to operation. Hematoxylin and eosin (H&E) stained slides were reviewed and 66 tumors were included. The tumor samples were fixed in 4% phosphate buffered formaldehyde directly after the operation and paraffin embedded. Malignancy grade, tumor size (diameter) and lymph node status at the time of diagnosis were evaluated. Tumors were classified according to World Health Organization (WHO) criteria and graded based on the recommendations of Elson and Ellis [16]. This study was approved by the Ethics Committee and previous informed consent was obtained from all patients for collection of breast cancer specimens in accordance with the guidelines of Qilu Hospital.

The patients were all female and the age at the time of the diagnosis ranged from 23 to 85 years (mean 46.3 years). The tumor size ranged from 1.0 to 7.8 cm in the greatest dimension with the mean size of 2.9 cm. Detailed patients' and disease characteristics were documented in Table 1. Patients were followed up from the date of surgery until death or the last observation (median follow-up, 71.4 months, ranging 13–120 months). At the time of the last follow-up, 44 patients (66.7%) were alive, 22 patients (33.3%) were dead from the disease.

### Tumor Ploidy Evaluation

The ploidy of 66 tumors was evaluated by measurement of DNA content using image cytometry on Feulgen stained sections as previously described [17]. DNA histograms were interpreted according to a modified subjective method. The normal control cells were given the value 2c, denoting the normal diploid DNA content, and all tumor-cell DNA values were expressed in relation to that. The histograms were divided into two groups. Cases with a major peak near the 2c region (1.8c–2.2c), and less than 10% cells exceeding 2.5c were denoted diploid tumor (D-tumor). DNA profiles with a stem line outside the diploid and tetraploidregion and distinctly scattered DNA values exceeding the tetraploid region (3.8c–4.2c) were classified as aneuploid tumor (A-tumor).

### FISH Procedure

High resolution QM-FISH was performed to detect gene copy number changes of CKS1BP7 and IGF1R in 66 cases. Probes were obtained from Professor Anders Zetterberg, Cancer Center Karolinska, Sweden, and labeled with Spectrum Green or Texas Red. The tissues were removed excess wax followed by dehydration in absolute alcohol. Antigenic recovery was performed through incubating the slides for 1 h at 80 °C in 0.1 M citric buffer (pH 6.0). A 10-min digestion with pepsin (1 mg/ml in 0.01 M HCL) was performed followed by fixation in 1% formaldehyde. The probes were dissolved in the hybridization mixture. Denaturation of probes and target DNAs were performed simultaneously at 90 °C for 10 min and each slide was

**Table 1** Patient and tumor characteristics in 66 patients with breast cancer

| Characteristics | Patients No. | CKS1BP7 Amp | *p* value | IGF1R Amp | *p* value |
|---|---|---|---|---|---|
| Age at diagnosis(y) | | | | | |
| ≦ 35y | 10 | 4 | 0.456 | 2 | 1.000 |
| > 35y | 56 | 15 | | 14 | |
| Tumor size[1] | | | | | |
| d ≦ 20mm | 22 | 4 | 0.252 | 5 | 1.000 |
| d > 20mm | 44 | 15 | | 11 | |
| Grades | | | | | |
| Low | 43 | 11 | 0.305 | 10 | 1.000 |
| High | 23 | 8 | | 6 | |
| Nodal Status | | | | | |
| N0 | 26 | 7 | 0.507 | 2 | 0.036 |
| N+ | 40 | 12 | | 14 | |
| ER | | | | | |
| Negative | 22 | 6 | 0.544 | 8 | 0.231 |
| Positive | 44 | 13 | | 8 | |
| PR | | | | | |
| Negative | 21 | 5 | 0.771 | 6 | 0.759 |
| Positive | 45 | 14 | | 10 | |
| HER-2 | | | | | |
| Negative | 51 | 14 | 0.748 | 11 | 0.493 |
| Positive | 15 | 5 | | 5 | |
| Ki67 | | | | | |
| Low | 27 | 4 | 0.053 | 2 | 0.009 |
| High | 39 | 15 | | 14 | |
| Tumor ploidy | | | | | |
| D | 22 | 2 | 0.019 | 2 | 0.066 |
| A | 44 | 17 | | 14 | |
| HR | | | | | |
| Negative | 22 | 6 | 0.544 | 8 | 0.231 |
| Positive | 44 | 13 | | 8 | |
| TNBC | | | | | |
| Non-TNBC | 55 | 16 | 0.608 | 11 | 0.083 |
| TNBC | 11 | 3 | | 5 | |

*d* diameter, *N0* node metastasis negative, *N+* node metastasis positive, *Amp* amplification, *D* diploidy, *A* aneuploidy, *HR* Hormone receptor, *TNBC* triple negative breast canccer

incubated in a moist chamber for hybridization at 47 °C overnight. After hybridization, slides were washed in 4 × SSPE for 10 min at 37 °C and 47 °C respectively. Nuclei were mounted and counterstained with 4′,6–diamino-2-phenylindole (DAPI; Vector Laboratories) followed by view in the fluorescence microscope.

Two researchers (YL and LL) independently carried out all investigations without knowledge of the clinicopathological data. Only signals in the tumor areas based on both a consecutive section stained by H&E and DAPI morphology were counted and evaluated. The exact signal number per nucleus was recorded, and at least 100 non-overlapping nuclei per sample were analyzed. Gene amplification was defined by the presence of more than 6 signals in more than 20% counted tumor cells.

### Immunostaining Evaluation

The immunostained slides of ER, PR, HER-2 and ki67 were reviewed and reevaluated. Tumors were counted as positive for ER and PR if >1% of the nuclei of neoplastic cells showed definitive staining [18]. Ki67 status was scored low if <15% of the nuclei of neoplastic cells were positive, and high if ≥15% were positive [19]. HER-2 was scored according to ASCO/CAP HER2 clinical practice guideline [20]. Tumors were considered HER-2 positive if immunostaining was scored 3+, or HER-2/CEP17 > 2.0 or HER2/cell more than 6 by FISH.

### Statistical Analysis

All statistical analyses were performed using SPSS 16.0 (SPSS Inc. Chicago, IL). The Chi-square and Fisher exact test was used to evaluate the statistical significance between clinicopathological variables and CNAs of genes. Correlations were studied using the Spearman test. Survival of patients was analyzed using the Kaplan–Meier and Log-rank test. Statistical significance was set at 0.05 and all *p* value is two-sided.

## Results

### CKS1BP7 and IGF1R co-Amplified in DCIS and Invasive Breast Cancers

Among 66 breast cancers, 44 (66.7%) were A-tumors while 22 (33.3%) were D-tumors. Copy numbers of CKS1BP7 and IGF1R were identified by QM-FISH. As shown in Table 1, amplification of CKS1BP7 was found in 19 (28.8%) of all cases, mainly with low-level increase of gene copy number (dot like, <10 copies) (Fig. 1b, d). 16 samples (24.2%) had amplification of IGF1R (cluster pattern) (Fig. 1b, d). 9 tumors with gained CKS1BP7 had co-occurring IGF1R amplification. Significant correlation was observed between CKS1BP7 amplification and IGF1R amplification ($p = 0.01$). Then we compared gene CNAs between DCIS and invasive areas for each tumor, and identical genomic changes of CKS1BP7 and IGF1R were observed in all 66 tumors. Moreover, IGF1R amplification was noted in atypical hyperplasia of ductal epithelium (ADH) (Fig. 2).

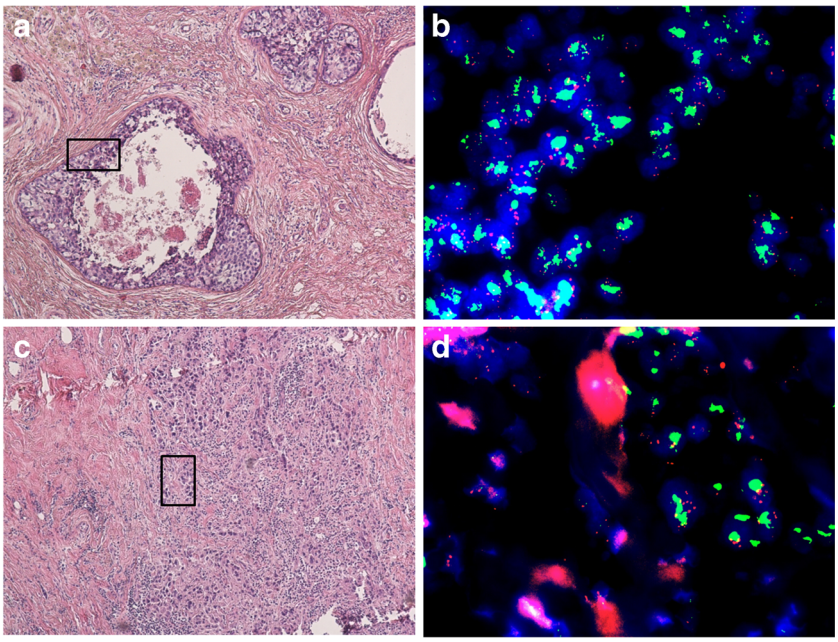


**Fig. 1** Detection of CKS1BP7 and IGF1R gene amplification by QM-FISH in DCIS and invasive breast cancer within the same samples. (**a**, **c**) Representative H&E images of DCIS (**a**) and invasive carcinoma (**c**) (×400). (**b**, **d**) Using QM-FISH, DCIS (**b**) and invasive carcinoma (**d**) show concurrent gain of CKS1BP7 and amplification of IGF1R.Red signals represent CKS1BP7 and green signals represent IGF1R(×630)

### Amplification of CKS1BP7 and IGF1R was Related to Clinicopathological Parameters, but Not Patients' Outcome

The increased frequency of CKS1BP7 amplification was observed in the aneuploid set relative to the diploid ($p = 0.019$). IGF1R amplification was weakly positively correlated to axillary lymph node metastasis ($p = 0.036$). Tumors with high ki67 index indicated more IGF1R amplification than tumors with low ki67 index ($p = 0.009$), as shown in Table 1. There was no significant correlation between amplification of these two genes and tumor size, hormone receptors status, HER-2 status as well as molecular subtypes. To evaluate the prognostic value of CNAs of CKS1BP7 and IGF1R, we performed survival analysis using Kaplan–Meier analysis. There was no significant correlation between CNAs of CKS1BP7 and IGF1R and patients' outcome ($p = 0.791$ and 0.983 respectively) (Fig. 3). Co-amplification of CKS1BP7 and IGF1R was not associated with clinicopathological characteristics and patients' outcome.

## Discussion

In the present study, FISH analysis revealed that 29% of 66 patients with breast cancer had amplification of CKS1BP7, implicating CKS1BP7, in spite of pseudogene, might play an important role in breast carcinogenesis. Pseudogenes have been defined as nonfunctional sequences of genomic DNA originally derived from functional genes. Pseudogenes pervade the genome, representing virtually every coding gene. Rather, pseudogenes have been investigated to exhibit functional roles, such as gene expression, gene regulation, generation of genetic (antibody, antigenic, and other) diversity [21]. It is thought that part of pseudogene sequences are copied into functional genes during genetic recombination, producing variants of the functional gene. For example, the eta-globin pseudogene is in fact functional, playing a regulatory role and assisting in "gene switching" between fetal and adult forms of hemoglobin [22]. Moreover a growing body of evidence strongly suggests that some pseudogenes are transcribed and contribute to cancer when dysregulated. Several transcribed

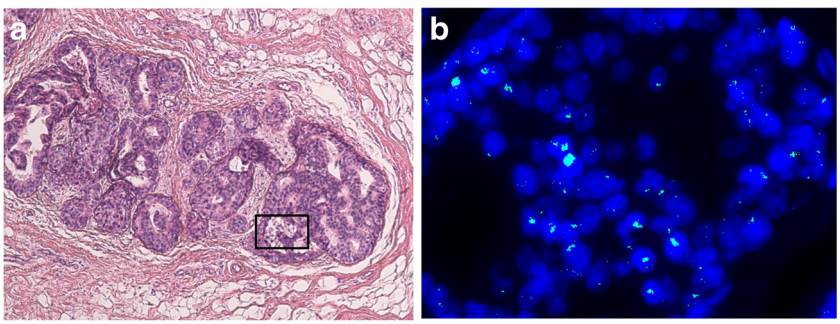


**Fig. 2** IGF1R gene amplification is observed in ductal atypical hyperplasia (×630)

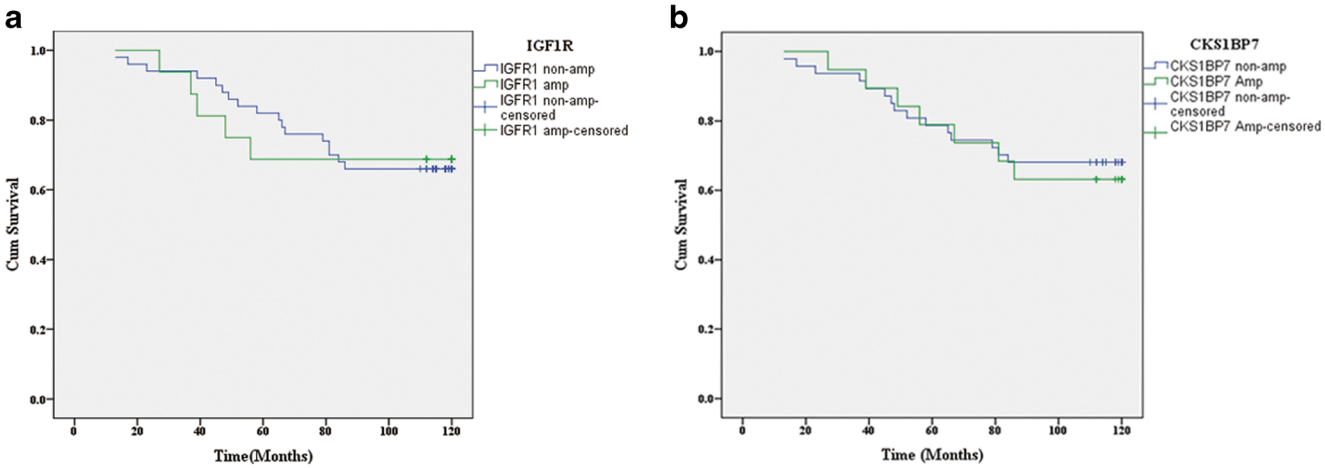


**Fig. 3** Kaplan–Meier analysis of overall survival curves stratified by CKS1BP7 and IGF1R amplification

pseudogenes, such as PTENP1, KRASP1, OCT4-pg4 and BRAFP1, are known to play a key role in the development of cancer and contribute to tumor progression [15]. To date, despite genomic sequence sharing considerable sequence identity with CKS1B, CKS1BP7 is known little. It is worth to study what the exact roles of CKS1BP7 are, and how CKS1BP7 functions in breast cancer. Our result showed frequent amplification of CKS1BP7 in invasive breast cancers indicating there might be overlapped roles between CKS1BP7 and CKS1B. However mainly low-level increase of gene copy number (<10 copies) was observed. To the best of our knowledge, this is first study that demonstrated CKS1BP7 amplified in cancerous samples.

CKS1B, the parent gene of CKS1BP, is one of the well-known members of the human CKS family and was identified in 1986 in fission yeast. Further biochemical studies revealed its vital roles in the regulation of cell division cycle [23, 24]. CKS1B is required for SCFSkp2-mediated ubiquitination and degradation of p27kip1, which is essential for the G1/S transition during the cell cycle [25, 26]. Besides its well documented roles in the cell-cycle, the amplification and overexpression of CKS1B has been observed in various cancers, which attracted more attention. It has been reported by Shi et al. [14] that overexpression of CKS1B promoted myeloma cell drug-resistance through activating JAK/STAT3 signaling pathways which is also activated by IGF1. In turn, IGF1–IGF1R mediated upregulation of ubiquitin-related p27Kip1 degradation through increased expression of Skp2, the receptor component of an SCF ubiquitin ligase complex [13]. Moreover, IGF1 was found to regulate proliferation by decreasing G1 phase. These data shed light on an interesting link between CKS1B and IGF1- IGF1R pathway. As expected, co-amplified CKS1BP7 and IGF1R was observed in 9 tumors (13.6%, $p = 0.01$). Meanwhile, the detection performed by QM-FISH produces more convincing results than separate FISH analysis. Extensive in vitro and in vivo studies have implicated the roles of IGF1R in cancer development, maintenance, and progression through regulating cell growth, survival, and motility. We speculate that amplification of CKS1BP7 might be involved in mammary tumorigenesis combined with aberrant expression of IGF1R or there is a potential signaling cross-talk between these two genes in partial tumors.

To elucidate the exact role of CKS1BP7 and IGF1R amplification in breast cancer, we performed analysis to compare CNAs of both genes in concurrent DCIS and invasive components in all tumors. Gain of CKS1BP7 was also observed in DCIS in all cases, but not in ductal hyperplasia area, suggesting amplification of CKS1BP7 is an early event during carcinogenesis of breast cancer. IGF1R amplification was observed in ADH as well as in DCIS, which indicated IGF1R amplification might be involved in initiation of breast cancer and occur before morphological changes come out. Step study on large cases are needed, meanwhile cases with ADH and usual ductal hyperplasia should be included.

Then, abnormalities of CKS1BP7 and IGF1R in relation to clinicopathologic characteristics and patients' outcome were evaluated. Although part of data didn't reach the significant level, both amplified genes tended to be more prevalent in A-tumors and in tumors with high proliferation index (assessed by ki67). Majority of malignant tumors are aneuploid [10] and aneuploidy confers a moreaggressive character to tumor cells [27]. Our data indicated these amplicons may correlate with a more aggressive behaviour. The results are consistent the effect of CKS1B reported by other studies [28]. However, to get more convincing information, step studies with expanded sample size are needed. In addition, IGF1R amplification was also related with lymph node metastasis ($p = 0.036$), suggesting a poor prognosis for amplified IGF1R group. Amplification or overexpression of IGF1R has long been recognized for its role in tumorigenesis and growth [29, 30], and identified as poor prognostic factors in breast cancers [31].

However, neither CNAs of IGF1R and CKS1BP7 nor co-amplification of these two genes was found correlated with patients' outcome in present study, which may due to small patient cohort.

In summary, our study investigated CKS1BP7 amplifications as a frequent event in breast cancer and found a high frequency of concurrent CKS1BP7 and IGF1R. Our data indicate CKS1BP7 has potential as a candidate oncogene and confirm the role of pseudogenes in tumorigenesis. We speculate CKS1BP7 gene functions in the pathogenesis and development of breast cancer through interaction with IGF1R signaling pathways and correlates with more aggressive behavior of the tumors. Thus, CKS1BP7 and IGF1R may serve as valuable biomarkers for early detection and prognosis evaluation in breast cancer. However, there was more about CKS1BP7 to study as a pseudogene than currently known likely function, and more detailed experimental work is required to determine how CKS1BP7 and IGF1R interact including in vitro study and detection of CKS1BP7 and IGF1R at protein and mRNA levels.

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