Cullin1 is a novel marker of poor prognosis and a potential therapeutic target in human breast cancer

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Background: To investigate the role of Cullin1 (Cul1) in the development of breast cancer, we examined the expression of Cul1 in breast cancer tissues and analyzed the correlation between Cul1 expression and clinicopathologic variables and patients survival.

Patients and methods: We evaluated the Cul1 expression by immunohistochemistry using a tissue microarray (TMA) which includes 393 breast cancer tissues. We also studied the role of Cul1 in breast cancer cell proliferation, migration and invasion by carrying out CCK8 cell proliferation assay, cell migration and invasion assay.

Results: The Cul1 expression was significantly correlated with breast cancer histology grade (P = 0.000), estrogen receptor status (P = 0.001), progesterone receptor status (P = 0.001) and human epidermal growth factor receptor 2 status (P = 0.002). Furthermore, we showed a strong correlation between high Cul1 expression and worse 5-year overall and disease-specific survival rates in breast cancer patients (P = 0.026 and P = 0.015, respectively). Finally, we found that Cul1 knockdown inhibits cell proliferation, migration and invasion abilities.

Conclusions: Cul1 overexpression is significantly correlated with breast cancer progression and predicts worse survival. Cul1 regulates breast cancer cell proliferation, migration and invasion.

Key words: breast cancer, cullin1, migration, invasion, prognostic, proliferation

Introduction

Breast cancer is the most common cancer in women, and it’s survival rate falls from 90% for localized to 20% for metastatic disease [1]. Tumor metastasis occurs by a complex series of events including cell proliferation, migration, invasion, adhesion and vessel formation [2]. Although tumor invasion and metastasis contribute to the great majority of breast cancer deaths, our understanding of the underlying molecular mechanisms is still limited [3].

A major pathway controlling protein degradation is the ubiquitin-proteasome system [4]. The attachment of ubiquitin to target proteins is mediated by at least three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3) [5]. Dysfunction of E3 ubiquitin ligases contributes to abnormal cell growth and differentiation [6]. As the biggest family of E3 ubiquitin ligases, the Skp1/Cullin/Rbx1/F-box protein (SCF) complexes ubiquitinate a broad range of proteins involved in cell-cycle...
survival. In addition, we further investigated the role of Cul1 in
expression and clinicopathologic variables and patient
tissue microarray (TMA) of human breast cancer patients and
human breast cancer.

It has been reported that loss of Cul1 results in early
embryonic lethality and deregulation of cyclin E [9]. Cul1
expression is increased in early stages of melanoma and
increased Cul1 expression promotes melanoma cell
proliferation through regulating p27 expression [10, 11].
In addition, Min et al. reported that Cul1 expression was
correlated with histological grade, negative ER and positive
HER2, and also shows a significant association with poor
overall survival in breast cancer [12]. Previously, we have
shown that overexpression of Cul1 is associated with poor
prognosis of patients with gastric cancer [13]. In this study, we
further investigated the role of Cul1 in the development of
human breast cancer.

To evaluate the function of Cul1 in breast cancer, we used a
TMA (tissue microarray) of human breast cancer patients and
immunohistochemistry to analyze the correlation between
Cul1 expression and clinicopathologic variables and patient
survival. In addition, we further investigated the role of Cul1 in
breast cancer cell proliferation, migration and invasion.

materials and methods

patient specimens

The study material consists of a series of 393 consecutive cases of primary
invasive breast carcinoma, from The First Affiliated Hospital of Nanjing
Medical University, between 1996 and 2005. All these patients were treated
with surgery only or with postoperative adjuvant therapy. The patients’
clinicopathologic information was obtained from the archive of the
pathology department and confirmed by the medical record of the hospital.
The histologic grade was assessed using Bloom–Richards classification.
Five-year clinical follow-up results were available for 224 patients. The use
of these specimens and data for research purposes was approved by the
Ethics Committee of the Hospital.

immunohistochemistry of TMA

Immunohistochemistry was carried out according to the avidin
biotinylated-HRP complex method. Briefly, sections were deparaffinized,
rehydrated and retrieved in water bath, using citrate buffer for 20 min. The
TMA slides were incubated with a monoclonal mouse anti-Cul1 antibody
(Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, and
diaminobenzidine (Zhongshan Biotech, Beijing, China) was used to
produce a brown precipitate. Negative controls were obtained by
substituting primary antibodies with non-immune serum.

evaluation of immunostaining

The evaluation of Cul1 staining was blindly and independently examined
by two pathologists. Positive Cul1 immunostaining is defined as
cytoplasmic with or without nuclear staining and graded according to both
the intensity and percentage of cells with positive staining. The Cul1
staining intensity was scored 0–3 (0 = negative; 1 = weak; 2 = moderate;
3 = strong). The percentage of Cul1-positive stained cells was also scored
into four categories: 1 (0%–25%), 2 (26%–50%), 3 (51%–75%) and 4
(76%–100%). The level of Cul1 staining was evaluated by the
immunoreactive score (IRS), which is calculated by multiplying the scores
of staining intensity and the percentage of positive cells. Based on the IRS,
the Cul1 staining pattern was defined as negative (IRS: 0), weak (IRS: 1–2),
moderate (IRS: 3–6) and strong (IRS: 8–12).

cell culture and transfection

Two human breast carcinoma cell lines MDA-MB-231 and BT-549 were
purchased from the Shanghai Institute of Biochemistry and Cell Biology
(Shanghai, China). Cells were cultured in RPMI 1640 medium and
supplemented with 10% fetal bovine serum (Invitrogen, Shanghai, China).
Cells were grown to 50% confluence before small-interfering RNA (siRNA)
transfection. Nonspecific control siRNA (Qiagen, Mississauga, ON,
Canada) or Cul1 siRNA (Dharmacon, Lafayette, CO) was transfected by
siLentFect Lipid Reagent (Bio-Rad, Hercules, CA) according to the
manufacturer’s instructions.

western blot analysis

Western blots were carried out as previously described [14]. The following
antibodies were used for western blot: mouse anti-Cul1 (Santa Cruz
Biotechnology), mouse anti-cyclin A, anti-cyclin E, anti-p21, anti-p27,
rabbit anti-cyclin D1, anti-MMP-2, anti-TIMP-2 (all from Cell Signaling
Technology, Beverly, MA) and mouse anti-β-actin (Boster Biotechnology,
Wuhan, China).

cell proliferation assay

Cellular proliferation was analyzed using a WST-8 cell counting kit-8
(Beyotime, Nantong, China). 3 × 10^5 cells were seeded in 96-well plates and
incubated for 24 h, 48 h, 72 h and 96 h. Ten microliter CCK-8 solution was
added to each well and the cultures were incubated at 37°C for 1 h.
Absorbance at 450 nm was measured using an ELX-800 spectrometer
reader (Bio-Tek Instruments, Winooski).

cell-cycle analysis

Thirty-six hours after transfection, the cells were treated with 1 μg/ml
aphidicolin. After 12 h, the cells were incubated in fresh medium
containing 50 ng/ml nocodazole for 0, 3 or 6 h. Then, the cells were fixed
with 70% cold ethanol at 4°C overnight, and stained with 40 μg/ml
propidium iodide in hypotonic fluorochrome buffer for 30 min. Samples
were then analyzed using a FACSCanto flow cytometer (BD Biosciences,
San Jose, CA).

migration assay

Cell migration was determined by using a modified two-chamber migration
assay with a pore size of 8 μm. 1 × 10^5 cells were seeded in serum-free
medium in the upper chamber. After 12 h incubation at 37°C, cells in the
upper chamber were carefully removed with a cotton swab and the cells
that had traversed the membrane were fixed in methanol, stained with
Trypan Blue and counted.

invasion assay

The invasion assay was carried out using a modified two-chamber plates
with a pore size of 8 μm. The transwell filter inserts were coated with
matrigel. 2 × 10^5 cells were seeded in serum-free medium in the upper
chamber. After 24 h incubation at 37°C, the cells in the upper chamber
were carefully removed with a cotton swab and the cells that had traversed
the membrane were fixed in methanol, stained with Trypan Blue and counted.
gelatin zymography

Gelatin zymography was carried out as previously described [14]. Thirty-six hours after transfection, cells were incubated in serum-free medium for 24 h. The proteins in the conditioned medium were concentrated with Ultracel-30 k centrifugal filters (Millipore, Billerica, MA). Sixty micrograms of the proteins were loaded for the gelatin zymography.

statistical analysis

For a TMA, statistical analysis was carried out with SPSS 20.0 software (SPSS, Chicago, IL). The association between Cul1 staining and the clinicopathologic parameters of the breast cancer patients was evaluated by a $\chi^2$ test. The Kaplan–Meier method and log-rank test were used to evaluate the correlation between Cul1 expression and patient survival. For CCK-8 cell proliferation assays, Student $t$-test was used. Differences were considered significant when $P < 0.05$.

results

correlation of Cul1 staining with clinicopathologic parameters

To investigate Cul1 expression in breast cancer, immunohistochemistry was carried out in TMA slides (Figure 1). Of the 393 breast cancer analyzed, negative and positive Cul1 staining were 28.2% (111/393) and 71.8% (282/393), respectively (Table 1). We then analyzed the relationship between Cul1 expression and characteristics of the breast carcinomas, and found that Cul1 staining was dramatically increased in histology grade II and III compared with histology grade I ($P = 0.000$, $\chi^2$ test, Figure 2A). Interestingly, we also found that increased Cul1 expression is significantly correlated with negative ER ($P = 0.001$, $\chi^2$ test, Figure 2B), negative PR ($P = 0.001$, $\chi^2$ test, Figure 2C) and positive HER2 ($P = 0.002$, $\chi^2$ test).

Table 1. Cul1 staining and clinicopathologic characteristics of 393 breast cancer patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cul1 staining</th>
<th></th>
<th>Total</th>
<th>$P^*$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Negative (%)</td>
<td>Positive (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$\leq$50</td>
<td>56 (30.4)</td>
<td>128 (69.6)</td>
<td>184</td>
<td>0.372</td>
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<tr>
<td>&gt;50s</td>
<td>55 (26.3)</td>
<td>154 (73.7)</td>
<td>209</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (&lt;2)</td>
<td>22 (31.0)</td>
<td>49 (69.0)</td>
<td>71</td>
<td>0.735</td>
</tr>
<tr>
<td>T2 (2-5)</td>
<td>78 (27.8)</td>
<td>203 (72.2)</td>
<td>281</td>
<td></td>
</tr>
<tr>
<td>T3 (&gt;5)</td>
<td>10 (33.3)</td>
<td>20 (66.7)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>54 (30.3)</td>
<td>124 (69.7)</td>
<td>178</td>
<td>0.290</td>
</tr>
<tr>
<td>Positive</td>
<td>46 (24.9)</td>
<td>139 (75.1)</td>
<td>185</td>
<td></td>
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<td>Histology grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>51 (78.5)</td>
<td>14 (11.5)</td>
<td>65</td>
<td>0.000</td>
</tr>
<tr>
<td>II</td>
<td>57 (24.4)</td>
<td>177 (75.6)</td>
<td>234</td>
<td></td>
</tr>
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<td>III</td>
<td>3 (3.3)</td>
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<tr>
<td>Ductal</td>
<td>94 (26.9)</td>
<td>256 (73.1)</td>
<td>350</td>
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<tr>
<td>Lobular</td>
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<td>9 (60.0)</td>
<td>15</td>
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<tr>
<td>Other</td>
<td>11 (39.3)</td>
<td>17 (61.7)</td>
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<td>ER status</td>
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<tr>
<td>Negative</td>
<td>12 (14.8)</td>
<td>69 (85.2)</td>
<td>81</td>
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<tr>
<td>Positive</td>
<td>44 (37.3)</td>
<td>74 (62.7)</td>
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<td>PR status</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>16 (16.7)</td>
<td>80 (83.3)</td>
<td>96</td>
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<td>Positive</td>
<td>40 (38.8)</td>
<td>63 (61.2)</td>
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<td>HER2 status</td>
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</tr>
<tr>
<td>Negative</td>
<td>17 (53.1)</td>
<td>15 (46.9)</td>
<td>32</td>
<td>0.002</td>
</tr>
<tr>
<td>Positive</td>
<td>33 (23.1)</td>
<td>110 (76.9)</td>
<td>143</td>
<td></td>
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</table>

*P values are obtained from $\chi^2$ test.

Figure 1. Representative images of Cul1 immunohistochemical staining in breast cancer tissue. (A and C) Negative staining in breast cancer tissue. (B and D) Positive staining in breast cancer tissue. Top panel, magnification ×200; bottom panel, magnification ×400.
We did not find any significant correlations between Cul1 expression and other clinicopathologic variables, including patient age, tumor size, lymph node metastasis and histology type.

**increased Cul1 expression with poor patient survival**

We then analyzed whether Cul1 expression was associated with the survival of patients by Kaplan–Meier survival analysis. Our data revealed that positive Cul1 staining correlated with both 5-year overall and disease-specific patient survival ($P = 0.026$ and $P = 0.015$, respectively, log-rank test; Figure 2E and F). The univariate Cox regression analyses revealed that Cul1 expression was significantly associated with overall or disease-specific survival in breast cancer patients ($P = 0.029$ and $P = 0.018$, respectively, supplementary Table S1, available at *Annals of Oncology* online). In supplementary Table S2, available at *Annals of Oncology* online, the multivariate Cox regression model indicated that expression of Cul1 is an independent prognostic marker for both overall and disease-specific survival ($P = 0.025$ and $P = 0.016$, respectively).

**Cul1 regulates cell proliferation in breast cancer cells**

Small-interfering RNA was used to knock down Cul1 expression in both MDA-MB-231 and BT-549 cells for an *in vitro* study (Figure 3A). In a CCK-8 cell proliferation assay, we found that the ability of cell proliferation was drastically decreased after Cul1 knockdown in both cell lines (Figure 3B). We then examined whether knockdown of Cul1 induced inhibition of breast cancer cell growth is due to cell-cycle arrest using fluorescence activate cell sorting. Our data showed that Cul1 knockdown cells have significantly higher G1 population than control cells in both the cell lines (Figure 3C). Western blot results showed that the mechanism of Cul1 regulation on cell-cycle progression is due to increased expression of p21 and p27, and decreased expression of cyclin A, cyclin D1 and cyclin E after Cul1 knockdown (Figure 3D).
silencing of Cul1 inhibits breast cancer cells migration and invasion in vitro

We next investigated the role of Cul1 in migration and invasion of breast cancer cells by a migration assay and matrigel invasion assay. In a cell migration assay, our data showed that Cul1 knockdown decreased cells migration ability of MDA-MB-231 and BT-549 cells by 83% and 77%, respectively (Figure 4A). In a cell invasion assay, silencing of Cul1 inhibited cell invasive ability of MDA-MB-231 and BT-549 cells by 84% and 79%, respectively (Figure 4B). Gelatin zymography data revealed that MMP-2 enzyme activity was significantly suppressed after knockdown of Cul1 in MDA-MB-231 and BT-549 cells (Figure 4C). Western blot results showed that the inhibition of the MMP-2 protein level is due to the increased expression of TIMP-2 after Cul1 knockdown in both cell lines (Figure 4D).

discussion

Histological grade, ER, PR and HER2 status all influence breast cancer prognosis and probability of response to systemic therapies [15]. Endocrine therapy is probably the most important systemic therapy for hormone receptor-positive breast cancer [16]. The loss of ER expression is thought to be involved in endocrine therapy resistance. Investigation surrounding the ER-negative phenotype has suggested that hyperactivation of the mitogen-activated protein kinase (MAPK) pathway reversibly inhibits the expression of the ER [17]. Although its role in breast cancer has not been addressed, it is possible that overexpression of Cul1 may contribute to a hyperactivated MAPK pathway in breast cancer. Patients with a HER2-positive breast cancer diagnosis often experience more aggressive tumor progression and an inferior prognosis [18]. Our studies showed a significant correlation between Cul1 and HER2. But interactions between Cul1 and HER2 signaling pathways remain poorly characterized.

Cyclins, their associated kinases (CDKs), and cyclin-inhibitory proteins are integral components in the coordinated progression of the cell cycle. We found that the ability of cell proliferation was drastically decreased after Cul1 knockdown in breast cancer cells by an arrest in the cell-cycle progression at the G1 to S transition. Cyclin A-CDK2, cyclin D1-CDK2 and cyclin E-CDK2 complex operates at the G1 to S transition [19].
Cyclin/CDK complexes are precisely regulated by Cip/Kip proteins, such as p21 and p27, which can bind and inhibit CDK activity at the G1/S cell-cycle checkpoint [20]. Our data showed that knockdown of Cul1 simultaneously up-regulated p27 and p21 expression, which subsequently inhibits the expression of cyclin A, cyclin D1 and cyclin E. However, it remains to be elucidated how Cul1 regulates p27 and p21 expression in breast cancer cell proliferation.

Figure 4. Knockdown of Cul1 inhibits breast cancer cells migration and invasion abilities. (A) Cell migration assay was carried out after Cul1 knockdown in MDA-MB-231 and BT-549 cells. (B) A matrigel cell invasion assay was carried out after Cul1 knockdown in MDA-MB-231 and BT-549 cells. (C) Gelatin zymography analysis of the relative enzyme activities of MMP-2 in Cul1 knockdown and control siRNA group for both MDA-MB-231 and BT-549 cell lines. (D) Western blot analysis of the relative protein levels of Cul1, TIMP-2 and MMP-2 in Cul1 knockdown and control siRNA group for both MDA-MB-231 and BT-549 cell lines. All experiments were carried out in triplicate. Data are shown as mean ± SE. ***P < 0.001.
Degradation of basement membranes and stromal ECM is crucial for invasion and metastasis of malignant cells. MMPs belong to the family of proteolytic zinc- and calcium-containing enzymes, which are responsible for degrading most components of ECM and basement membrane [21]. The activity of MMPs is controlled by interaction with the TIMPs. Giannelli et al. showed that the imbalance between MMPs and TIMPs is responsible for cancer metastasis [22]. However, an association between Cul1 and migration and invasion abilities in vitro has never been reported. Our results showed knockdown of Cul1 in breast cancer cells resulted in significantly inhibited cell migration and invasion abilities, and this was due to the MMP-2/TIMP-2 imbalance.

In conclusion, our results demonstrate that increased Cul1 expression is significantly correlated with poor prognosis of patients with breast cancer. Cul1 regulates breast cancer cell proliferation through cell-cycle control. Further demonstrate that imbalance between MMP-2 and TIMP-2 after Cul1 knockdown greatly contributed to the reduced cell migration and invasion abilities in breast cancer cells. Thus, Cul1 might be an important marker and a therapeutic target for breast cancer.

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disclosure
The authors have declared no conflicts of interest.

references