RPN11 deubiquitinase promotes proliferation and migration of breast cancer cells

GUOQING LUO¹,², NINGDONG HU², XU XIA², JINGJING ZHOU² and CHANGSHENG YE¹

¹Breast Center, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515; ²The First Zone of Cardiothoracic Department, Qingyuan People's Hospital, The Sixth Affiliated Hospital of Guangzhou Medical University, Qingyuan, Guangdong 511518, P.R. China

Received March 19, 2016; Accepted February 21, 2017

DOI: 10.3892/mmr.2017.6587

Abstract. The deubiquitinase enzyme RPN11 is involved in oncogenesis in various types of cancer. However, in breast cancer, the expression levels, prognostic relevance and biological function of RPN11 remains unclear. In the present study, RPN11 expression levels in breast cancer tissues and adjacent non-tumor tissues were determined by reverse transcription-quantitative polymerase chain reaction and immunohistochemical staining, and the association of RPN11 with clinicopathological features of breast cancer was evaluated. RPN11 expression was upregulated in breast cancer tissues compared with healthy tissues. Additionally, high expression levels of RPN11 may be an indicator of poor prognosis, as validated by a breast cancer cohort from the Gene Expression Omnibus database. Knockdown of RPN11 in MDA-MB-231 and T47D cells significantly reduced cell proliferation and enhanced G0/G1 arrest and apoptosis. Exogenous overexpression of RPN11 in MCF7 and Hs578T cells promoted cell growth and inhibited apoptosis. In addition, knockdown of RPN11 abrogated cell migration and reduced epithelial-mesenchymal transition. In conclusion, these findings suggested that RPN11 may function as an oncogene and its upregulation in breast cancer suggests that it may be a therapeutic target.

Introduction

Breast cancer (BC) is the most frequently diagnosed malignancy and is one of the leading causes of mortality in women, with nearly a quarter of million new cases occurring in the United States in 2016 (1,2). The heterogeneity of breast cancer is a primary factor that contributes to the intractable clinical treatments. For example, hormone therapy is available for luminal A or B breast cancers that are estrogen (ER)/progesterone (PR) positive. However, there are limited available treatments for breast cancer that is triple-negative for ER, PR and human epidermal growth factor receptor 2 (HER2). Numerous molecular biomarkers and signaling pathways have been investigated, including aberrant activation of Akt and Wnt signaling pathways, in addition to changes in microRNAs and oncogenes (3-6). However, the mechanisms underlying the development of breast cancer require further understanding, particularly the development of malignant phenotypes.

The deubiquitinating enzyme RPN11, alternatively known as proteasome 26S subunit non-ATPase 14 or POH1, is a component of the 26S proteasome and reverses the effects of the ubiquitin-proteasome degradation system. RPN11 functions in diverse biological processes, including DNA repair, embryonic cell development and differentiation, programmed cell death and drug resistance (7-9). RPN11 has been reported to modulate the proliferation of tumor cells by regulating the phosphorylation of retinoblastoma protein and cyclin-dependent kinases (10). In addition, RPN11 was significantly upregulated in hepatocellular carcinoma and promoted tumorigenesis by stabilizing E2F transcription factor 1 (E2F1) and its target genes (11). Gallery et al (12) reported that the JAB1/MPN/Mov34 metalloenzyme motif of RPN11 was essential for cell viability. A previous study has suggested that RPN11 may regulate the ErbB2 receptor and c-jun ubiquitination, and enhance the transcriptional activity of microphthalmia-associated transcription factor (13-15). However, there is currently no evidence for the potential role of RPN11 in breast cancer development and treatment.

The present study reports that mRNA and protein expression levels of RPN11 were overexpressed in breast cancer tissue. RPN11 significantly promoted tumor cell proliferation and inhibited apoptosis. In addition, knockdown of RPN11 inhibited cell migration by suppressing epithelial-mesenchymal transition (EMT). Therefore, the results of the present study demonstrate that inhibition of RPN11 may serve as a treatment for breast cancer.

Materials and methods

Tissue specimens. A total of 59 breast cancer tissues and paired noncancerous tissues were obtained from the First Zone of Cardiothoracic Department, Qingyuan People’s Hospital (Qingyuan, China). The study was approved by the
Ethical Committee of Qingyuan People's Hospital and the written informed consent was obtained. The patients did not receive radiotherapy or chemotherapy prior to tissue collection. In addition, six pairs of fresh breast cancer tissues and adjacent noncancerous tissues were utilized for western blot analysis. Tissues were stored at -80°C prior to processing. A tissue microarray containing 142 cases of breast cancer specimens with complete clinicopathological features was utilized in this study and was obtained from Shanghai Outdo Biotech Co. Ltd. (cat. no. HBre-Duc150Sur-02; Shanghai, China). The remaining patients with breast cancer were obtained from GEO database (www.ncbi.nlm.nih.gov/).

**Cell lines.** MCF7, MDA-MB-231, Hs578T and T47D human cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C and 5% CO₂.

**Plasmid and siRNA transfection.** The pcDNA3.1-RPN11 plasmid was utilized for overexpression of RPN11 and was obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells at a density of 10⁵ cells/well were plated into the 6-well plate and transfected with pcDNA3.1-RPN11 or vector control using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For siRNA transfection, cells were transfected with a pool of three RPN11 siRNAs or a negative control siRNA. siRNAs targeting RPN11 were obtained from Chang Jing Bio-Tech, Ltd. (Changsha, China). The sequences were as follows: Negative control siRNA, 5'-UUC UCC GAA UCU GGC UCA G-3'; RPN11 siRNA-1, 5'-CAA GTT AAA GGA GGC CCA CAA G-3'; RPN11 siRNA-2, 5'-GCA AGA CAA GGG GAC CAA C-3'; RPN11 siRNA-3, 5'-TGT GTT TTC GAG GTC CAG C-3'. The cells were cultured for 48 h.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Primary antibodies, diluted at 1:1,000 were as follows: rabbit anti-RPN11 (catalog no. 10259-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), mouse anti-cyclin D1 (catalog no. sc-5286; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-cleaved PARP (catalog no. 5625) and mouse anti-snail (catalog no. 3895; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-E-cadherin (catalog no. 1702), rabbit anti-N-cadherin (catalog no. 2447), rabbit anti-cyclin D1 (catalog no. 2261) and rabbit anti-vimentin (catalog no. 2707; Epitomics, Burlingame, CA, USA).

**Cell viability assay.** The rate of cell proliferation was measured by MTT assay. The cells were transfected with RPN11 cDNA and vector as control, or siRNA and NC as control. Subsequently, the cells were seeded in 96-well plates to a density of 1,000 cells/well. MTT reagent (5 mg/ml) was added to the medium at various time points. Following 3 h incubation, the medium was discarded and DMSO was added to form a precipitate. The absorbance was measured at a wavelength of 540 nm.

**Flow cytometric assay.** Cells were seeded at 10⁴ fixed with 70% ethanol and stained with propidium iodide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and RNase (Takara Biotechnology Co., Ltd.) for 30 min. Subsequently, the cells were seeded in 96-well plates to a density of 1,000 cells/well. MTT reagent (5 mg/ml) was added to the medium at various time points. Following 3 h incubation, the medium was discarded and DMSO was added to form a precipitate. The absorbance was measured at a wavelength of 540 nm.

**Transwell assay.** Cells were starved in serum free medium for 24 h and seeded on the top chamber of a Transwell plate at 1x10⁴ cells/well. The bottom chambers were filled with medium supplemented with 20% FBS. After 48 h, cells remaining in the top chamber were carefully removed and the lower surface was fixed in 4% paraformaldehyde and stained...
with 1% crystal violet. Cells were counted and imaged in five random fields using a phase contrast microscope. Experiments were repeated at least three times.

**Immunohistochemical staining (IHC).** Tissue microarray slides were deparaffinized, rehydrated in ethanol and subjected to antigen retrieval by heating in 0.01 M citrate buffer (Beyotime Institute of Biotechnology, Haimen, China), to block endogenous peroxidases. Following washing in PBS and blocking with 5% bovine serum albumin (Sangon Biotech Co., Ltd.), the slides were incubated with a primary RPN11 antibody (cat. no. 12059-1-AP; ProteinTech Group, Inc.; 1:500 dilution) overnight at 4˚C. Following three washes in PBS, slides were incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. AR1022; 1:500; Boster Biological Technology, Ltd., Wuhan, China) for 1 h at room temperature and subsequently washed three times in PBS. Tissues were stained with diaminobenzidine and counterstained with hematoxylin and observed using a light microscope.

Tissues were evaluated by two pathologists and scored according to the percentage and intensity of staining, as previously described (17). Briefly, the percentage score was as followed: i) 0-5%; ii) 6-50%; iii) 51-75% and iv) 76-100%. The intensity score was as followed: i) No staining; ii) weak; iii) moderate and iv) strong. A final score from 1 to 16 was calculated by multiplying the percentage score by the intensity score. For each sample, ≤8=low expression and >8=high expression.

**Statistical analysis.** Statistical calculations were performed using SPSS version 21.0 software (IBM SPSS, Armonk, NY, USA). PROGgene version 2.0 software (Indiana University, Indianapolis, IN, USA) was used to evaluate the prognosis of indicated types of cancer. Clinical parameters were compared with RPN11 expression in tissue samples by the χ² test. Kaplan-Meier curves were utilized to determine the overall survival distribution. Student's t-test was performed to compare the significance between the control group and experimental groups. Data are expressed as the mean ± standard error of three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Elevated RPN11 expression in breast cancer is associated with poor prognosis.** To determine the potential role of RPN11 in breast cancer pathogenesis, the mRNA expression levels of RPN11 were determined in 59 cases of paired breast cancer tissue and adjacent non-cancer tissue samples. Greater expression levels of RPN11 were detected in breast cancer tissues compared with non-cancer adjacent tissue (P<0.001; Fig. 1A). Subsequently,
the GSE3744 dataset was analyzed, which contained 47 human breast tumor cases and 7 cases of non-cancerous tissues. The expression levels of RPN11 were greater in tumor tissues compared with healthy tissues (P<0.001; Fig. 1B). Western blot analysis demonstrated elevated RPN11 expression levels in six breast cancer tissues compared with adjacent non-cancerous tissues (Fig. 1C).

IHC was performed on tissue microarrays to measure RPN11 expression in 142 tissues derived from breast cancer patients, whose 5-year follow-up data were available. Low levels of RPN11 were detected in adjacent non-cancerous tissues, whereas high levels of RPN11 staining were observed in breast cancer tissues (Fig. 1D). In addition, RPN11 expression was associated with the clinical tumor stage (P<0.05; Table I). However, there was no association between RPN11 expression and age, tumor size, lymphatic vessel invasion, T classification or N classification.

Patients with high RPN11 expression exhibited significantly poorer overall survival, as determined by Kaplan-Meier analysis with log-rank test (log-rank=5.475; P=0.019; Fig. 1E). This was supported by the GSE1456 dataset, which was analyzed using PROGgene version 2.0 software (Indiana University, Indianapolis, IN, USA) (18,19). High expression of RPN11 led to an enhanced degree of apoptosis (Fig. 2B) and suppression of the cell cycle, compared with cells transfected with negative control siRNA, suggesting that RPN11 may serve a role in oncogenesis (Fig. 2A). In addition, the effect of RPN11 knockdown on apoptosis and the cell cycle was investigated. Flow cytometric analysis suggested that knockdown of RPN11 induced G0/G1 arrest (Fig. 2C). Knockdown of RPN11 induced G0/G1 arrest (Fig. 2C). In addition, cyclin D1, a marker of the cell cycle, and cleaved PARP expression, a marker of apoptosis, were measured by western blot analysis. Knockdown of RPN11 enhanced cleaved-PARP and reduced cyclin D1 expression levels (Fig. 2D). This suggested that knockdown of RPN11 inhibited cell proliferation and induced apoptosis.

To further evaluate the role of RPN11 in breast cancer, RPN11 was exogenously overexpressed in MCF7 and Hs578T cells. Overexpression of RPN11 promoted cell growth (P<0.05; Fig. 3A) and cell cycle progression (Fig. 3B); however, apoptosis was reduced compared with the vector control (P<0.05).

### Table I. RPN11 expression status and clinicopathological features of breast cancer tissue samples.

<table>
<thead>
<tr>
<th>Clinicopathological feature</th>
<th>No. of cases</th>
<th>RPN11 expression</th>
<th>( \chi^2 )</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \leq 55 )</td>
<td>64</td>
<td>Negative</td>
<td>0.333</td>
<td>0.564</td>
</tr>
<tr>
<td>( &gt;55 )</td>
<td>78</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt; 2 )</td>
<td>19</td>
<td>Negative</td>
<td>2.849</td>
<td>0.241</td>
</tr>
<tr>
<td>( \geq 2 ) and (&lt; 5 )</td>
<td>100</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \geq 5 )</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphatic vessel invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>70</td>
<td>Negative</td>
<td>2.886</td>
<td>0.089</td>
</tr>
<tr>
<td>No</td>
<td>72</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( T_0-1 )</td>
<td>38</td>
<td>Negative</td>
<td>3.071</td>
<td>0.080</td>
</tr>
<tr>
<td>( T_2-4 )</td>
<td>104</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( N_0-1 )</td>
<td>110</td>
<td>Negative</td>
<td>3.511</td>
<td>0.061</td>
</tr>
<tr>
<td>( N_2-3 )</td>
<td>32</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>24</td>
<td>Negative</td>
<td>6.435</td>
<td>0.040*</td>
</tr>
<tr>
<td>II</td>
<td>80</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III+IV</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05.

RPN11 promotes cell growth and inhibits apoptosis in breast cancer cells. The effect of RPN11 inhibition on the MDA-MB-231 and T47D breast cancer cell lines was investigated. Knockdown of RPN11 reduced the proliferation of MDA-MB-231 and T47D cells compared with cells transfected with negative control siRNA, suggesting that RPN11 may serve a role in oncogenesis (Fig. 1B). In addition, the effect of RPN11 knockdown on apoptosis and the cell cycle was investigated. Flow cytometric analysis suggested that knockdown of RPN11 induced G0/G1 arrest (Fig. 2C). Knockdown of RPN11 induced G0/G1 arrest (Fig. 2C). In addition, cyclin D1, a marker of the cell cycle, and cleaved PARP expression, a marker of apoptosis, were measured by western blot analysis. Knockdown of RPN11 enhanced cleaved-PARP and reduced cyclin D1 expression levels (Fig. 2D). This suggested that knockdown of RPN11 inhibited cell proliferation and induced apoptosis.
This data suggested that RPN11 may have oncogenic potential and may be a therapeutic target. **RPN11 knockdown reduces breast cancer cell migration.** Knockdown of RPN11 resulted in reduced cell migration in breast cancer cells.
breast cancer cells compared with the negative control, as
determined by a Transwell assay (Fig. 4A). EMT is a key step
in tumor cell migration (20). Therefore, genes associated with
EMT were measured by RT-qPCR following RPN11 knock-
down. The level of mRNA encoding the epithelial marker
E-cadherin was increased, whereas the levels of mRNA
encoding the mesenchymal markers N-cadherin and vimentin
were reduced following knockdown of RPN11, compared with
the negative control (Fig. 4B). The mRNA expression levels
of EMT-associated transcriptional regulators, Snail and Slug,
were reduced following RPN11 knockdown. In addition,
western blot analysis corresponded with the RT-qPCR data
(Fig. 4C). Collectively, the results suggested that overexpres-
sion of RPN11 in breast cancer may promote cancer cell
migration.

Discussion

The mortality rate of breast cancer has declined due to
improvements in earlier diagnosis and adjuvant therapies.
Novel prognostic markers remain to be identified, carcino-
embryonic antigen and carcinoma antigen 15-3 are widely
recognized as tumor markers of breast cancer; however,
the sensitivity and precision of the assays utilized for their
detection requires further investigation (21-23). Therefore, it
is necessary to identify novel markers for the diagnosis and
treatment of patients with breast cancer.

The results of the present study indicated that RPN11 was
significantly upregulated in breast cancer tissues compared
with adjacent non-tumor tissues, and high expression of RPN11
was associated with the clinical tumor stage. Patients with high
RPN11 expression levels had significantly poorer outcomes,
which suggested that RPN11 may be a prognostic factor for
patients with breast cancer. To validate these findings, the
GSE1456 breast cancer cohort was analyzed. Results revealed
that patients with high RPN11 expression had poor survival
outcomes. In addition, in vitro experiments demonstrated that
knockdown of RPN11 reduced proliferation and induced cell
cycle arrest and apoptosis in breast cancer cells. By contrast,
overexpression of RPN11 in breast cancer cells promoted cell
growth and inhibited apoptosis.

Recently, RPN11 has been reported to function as an onco-
gene in a number of cancer types, including hepatocellular
carcinoma and ovarian cancer (11). Wang et al (11) reported
that RPN11 acts as a deubiquitinase enzyme and stabilizes
the expression of E2F1 by removal of the polyubiquitin chain,

---

Figure 3. RPN11 overexpression promotes cell growth and inhibits apoptosis. (A) Cell proliferation assay was performed following transfection with the vector
control or the RPN11 plasmid in MCF7 and Hs578T cells. RPN11 overexpression was confirmed by western blot analysis. (B) Analysis of the cell cycle and
(C) apoptosis in MCF7 and Hs578T cells following RPN11 overexpression, as determined by flow cytometry. Data are expressed as the mean ± standard error
of three independent experiments. *P<0.05. OD, optical density.
resulting in abnormal cell proliferation and tumorigenesis. In addition, it was demonstrated that expression of RPN11 was associated with the expression of survivin and forkhead box M1, both of which are considered to be oncogenes. Byrne et al. (10) suggested that knockdown of RPN11 in HeLa cells enhanced cell cycle arrest and senescence by downregulation of cyclin B1-cell division cycle 25C and cyclin D1, and upregulation of p21, which was consistent with the findings of the present study. Furthermore, RPN11 promoted the double-strand DNA break response by processing of the polyubiquitin chain and recruiting p53 binding protein 1 to the site of DNA damage (24). RPN11 may specifically cleave lysine 63-linked polyubiquitin chains, which are critical for the regulation of substrates (25). Therefore, the downstream targets of RPN11 require further investigation.

The results of the present study suggested that RPN11 promotes cell migration by inducing EMT. Despite there being no clinical association between RPN11 expression and N classification, knockdown of RPN11 reduced cell migration by inducing mesenchymal-epithelial transition (MET). In EMT, cells lose their adhesion and epithelial features and switch to a mesenchymal phenotype, which is an indicator of cancer metastasis (26). In the present study, as the relative expression of RPN11 was higher in MCF-7 and Hs578T compared with MDA-MB-231 and T47D (data not shown), MCF-7 and H578T were used for the overexpression of RPN11 and MDA-MB-231/T47D cells were used for the knockdown. The Transwell assay suggested that knockdown of RPN11 partially abrogates the migration of breast cancer cells. To verify this, the expression levels of EMT-associated genes were measured, including E-cadherin, FN1, Snail and Slug. Results suggested that inhibition of RPN11 induced MET.

In conclusion, overexpression of RPN11 in breast cancer tissues was associated with an advanced clinical stage. Patients with tumors with high expression of RPN11 had worse prognosis. These findings suggest a role of RPN11 in breast cancer development, progression, cell proliferation and migration by inducing EMT. The data suggest that RPN11 may be a therapeutic target for breast cancer.
References


