Ribosomal L22-like1 (*RPL22L1*) Promotes Ovarian Cancer Metastasis by Inducing Epithelial-to-Mesenchymal Transition

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Abstract

Double minute chromosomes (DMs) have important implications for cancer progression because oncogenes frequently amplified on them. We previously detected a functionally undefined gene amplified on DMs, Ribosomal L22-like1 (*RPL22L1*). The relationship between *RPL22L1* and cancer progression is unknown. Here, *RPL22L1* was characterized for its role in ovarian cancer (OC) metastasis and its underlying mechanism was examined. DNA copy number and mRNA expression of *RPL22L1* in OC cells was analyzed using data obtained from The Cancer Genome Atlas and the Gene Expression Omnibus database. An immunohistochemical analysis of clinical OC specimens was performed and the relationships between expression level and clinicopathological factors were evaluated. Additionally, in vivo and in vitro assays were performed to understand the role of *RPL22L1* in OC. *RPL22L1* expression was higher in OC specimens than in normal tissues, and its expression level was highly positively correlated with invasion and lymph node metastasis (*P* < 0.05). *RPL22L1* over-expression significantly enhanced intraperitoneal xenograft tumor development in nude mice and promoted invasion and migration *in vitro*. Additionally, *RPL22L1* knockdown remarkably inhibited UACC-1598 cells invasion and migration. Furthermore, *RPL22L1* over-expression up-regulated the mesenchymal markers vimentin, fibronectin, and α-SMA, reduced expression of the epithelial markers E-cadherin, α-catenin, and β-catenin. *RPL22L1* inhibition reduced expression of vimentin and N-cadherin. These results suggest that *RPL22L1* induces epithelial-to-mesenchymal transition (EMT). Our data showed that the DMs amplified gene *RPL22L1* is critical in maintaining the aggressive phenotype of OC and in triggering cell metastasis by inducing EMT. It could be employed as a novel prognostic marker and/or effective therapeutic target for OC.
Introduction

Ovarian cancer (OC) is the second most common gynecological malignancy and the first cause of death [1]. Cancer metastasis, rather than primary tumors, are responsible for most cancer deaths [2]. Owing to a lack of definitive early symptoms and appropriate markers for OC diagnosis at an early stage, the majority of patients are diagnosed with late-stage OC accompanied with metastasis, which typically has a 5-year survival rate of < 30% [3]. It is critical to understand the molecular mechanisms involved in OC metastasis, and to determine efficient, specific, and sensitive molecular targets that can be applied to metastasis diagnosis, prognosis, and individual treatment.

Double minute chromosomes (DMs) are cytogenetic hallmarks of gene amplification [4]. DMs appear in various kinds of human cancer cells, but not in normal cells [5]. As extra-chromosomal elements carrying amplifications of genomic DNA sequences, DMs contribute to cancer formation and progression, oncogenes are frequently present in the amplified sequences and the proteins they encode are often over-expressed [6]. Examples of genes amplified on DMs include MYC in colon cancer [7], MYCN in neuroblastoma [8], EGFR in gliomas [9], and EIF5A2 [10, 11] in ovarian cancer [12]. As DMs are vehicles of amplified genes including many oncogenes, functional studies of genes that are amplified on DMs is a good way to explore candidate oncogenes.

Our team previously identified 3q26.2 as an origin of DMs in the human ovarian cancer cell line UACC-1598, and a series of genes were co-amplified on the same ovarian DMs, including MYCN, EIF5A2, and RPL22L1 [13]. Both MYCN and EIF5A2 play important roles in cancer progression. However, the relationship between RPL22L1 and cancer is not known.

In this study, we showed that RPL22L1 is commonly over-expressed in clinical OC individuals and its expression level is strongly related to tumor invasion and metastasis. An in vivo experiment showed that forced expression of RPL22L1 promotes intraperitoneal xenograft tumor development in nude mice, and enhances cell migration and invasion in vitro. Furthermore, knocking it down with small interfering RNA (siRNA) inhibits migration and invasion in vitro. During this process, RPL22L1 over-expression resulted in elevated expression of mesenchymal markers such as vimentin and α-SMA, and decreased expression of epithelial markers, such as E-cadherin, α-catenin, and β-catenin, indicating that the induction of epithelial-to-mesenchymal transition (EMT) may explain the observed increases in motility and invasion ability for metastasis. Our data showed that RPL22L1 plays an important role in the process of OC metastasis.

Materials and Methods

Ethic statement

This study were approved by the Ethics Committee of Harbin Medical University with the following reference number, HMUIRB20150023. The ovarian cancer tissue microarrays (TMAs) for immunohistochemistry were purchased from US BIOMAX (ov951, ov1912, ov6161; Rockville, MD, USA) and Xin Chao (HOva-Can90PT-01; Shanghai, China). Both companies provided ethical statements to confirm that the local ethics committees approved their consent procedures, all participants provided their written informed consents and all efforts had been made to protect patient privacy and anonymity. The ethical statements provided by companies and the protocol of experiment had been checked carefully and approved by Ethics Committee of Harbin Medical University (HMUIRB20150023). Four-week-old female BALB/c mice (specific-pathogen-free) were purchased from SLAC (Shanghai, China) and housed in the Harbin Medical University Animal Laboratory. Mice were housed under standardized light-controlled...
conditions at room temperature (24°C) and 50% humidity, with free access to food and water. Animal experiments were performed in strict accordance with the recommendations in the Guidelines of Laboratory Animal Usage of Harbin Medical University. The protocol was approved by the Ethics Committee of Harbin Medical University (HMUIRB20150023) and all efforts were made to minimize suffering.

Cell lines and cell culture
Human ovarian cancer cell line UACC-1598, SKOV3, HO-8910, and HO-8910PM were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured following the methods described by the ATCC (Manassas, VA, USA), and were authenticated in 2012 at the Micro-read Genetics Company (Beijing, China) using a short tandem repeat analysis.

Preparation of metaphase spreads and fluorescence in situ hybridization (FISH) analysis
Cells were harvested for metaphase spread preparation according to the methods described in previous studies [14, 15] and were stained with Giemsa. Two BAC clones, GFP-RP11-355H10, which specifically covers the MYCN (amplified in UACC-1598 and located on the DMs [13]), and Cy3-RP11-726H11 for RPL22L1, were selected as DNA probes and hybridized to metaphase spreads of cells as described previously [16]. Chromosomes were counterstained with DAPI (4, 6-diamidino-2-phenylindole). Images were captured using a Leica DM5000 B fluorescence microscope (Wetzlar, Germany), and analyzed using the MetaMorph Imaging System (Universal Imaging Corporation, West Chester, NY, USA).

DNA copy number and gene expression analysis
The Oncomine DNA Copy Number Datasets (https://www.oncomine.org/resource/main.html) includes data deposited in The Cancer Genome Atlas (TCGA Ovarian 2 Dataset, http://tcga-data.nci.nih.gov/tcga/) was used to determine the differences in DNA copy number between OC and normal blood / ovarian tissues. 607 ovarian serous cystadenocarcinoma, 431 normal blood, and 130 normal ovary tissue samples were analyzed. Data were obtained through the following steps: type gene name: RPL22L1 in the search box and in the browse tree select Primary Filters > Datasets type > DNA Copy Number Datasets. The result of TCGA Ovarian 2 is directly in the first, and graph was on the left. Click on the histogram button on the upper left corner of the graph title to get a histogram graph. On the group filters above the graph title, click on the triangle symbol, in the menu selected "Cancer and Normal Type" to make the analysis grouped by cancer and normal samples. Data access requires an academic email account. Authors should be contacted for login information if necessary.

Two independent microarray gene expression datasets were used. Both datasets were generated using the Affymetrix Human Genome U133 Plus 2.0 Array platform (Santa Clara, CA, USA). The raw.CEL files for the two datasets were downloaded from the Gene Expression Omnibus (GEO) website (GSE27651 and GSE28450) [17, 18], and normalized using the RMA (robust multi-array average) algorithm [19].

qRT-PCR
Total RNA for each cell line was extracted using the High Pure RNA Isolation Kit (Roche, Basel-Stadt, Switzerland) following the manufacturer’s instructions. PrimeScript® RT Reagent Kit Perfect Real Time (Takara, Dalian, China) was used to reverse transcribed the total RNA.
The cDNA was quantified using LightCycler® 480 SYBR Green I Master (Roche) in a LightCycler™ 480 II Real Time System (Roche). The specific primers for human RPL22L1 and GAPDH were as follows: RPL22L1 forward primer, 5′-AGAAGGTAAAGTCATTG-3′ and reverse primer, 5′-ATCACGAAGATTGTTCTTC-3′; GAPDH forward primer, 5′-ATCACTGCCACCCAGAAGAC-3′ and reverse primer, 5′-TTTCTAGACGGCAGGTCAAG-3′. Expression of RPL22L1 in samples was normalized to that of GAPDH and the fold-change in expression was calculated using the 2^{-ΔΔCt} method.

Western blot analysis
Cells were lysed at 4°C in RIPA (8990, Thermo Fisher Scientific, Waltham, MA, USA). Cell lysates containing 40μg of total protein from each sample were loaded onto 12% sodium dodecyl polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking in 10% blocking solution (Roche), membranes were incubated overnight at 4°C with primary antibodies followed by 1-h incubation with anti-mouse/anti-rabbit secondary antibody (200-332-263/610-132-007, Rockland Immunochemicals, Limerick, PA, USA) at room temperature. Images were obtained using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and cropped using Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA), representative of five independent experiments. GAPDH was used as a control. The details of the primary antibodies are provided in the supplementary materials S1 Text.

Tissue microarray
The OC tissue microarrays (TMAs) were purchased from US BIOMAX (ov951, ov1912, ov6161; Rockville, MD, USA) and Xin Chao (HOva-Can90PT-01; Shanghai, China). Approval for this study was obtained before it was initiated from the local regional ethics committee. Written informed consent was obtained from all the participants.

Immunohistochemistry
Immunohistochemistry (IHC) studies were performed using PowerVision™ Two-Step Histostaining Reagent (Zhongshan Golden Bridge, Beijing, China) according to the manufacturer’s instructions. In brief, TMA sections were deparaffinized and rehydrated. For antigen retrieval, TMA slides were microwave-treated in 10mM citrate buffer (pH 6.0) for 8 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. The TMA slides were incubated with a 1:50 dilution of monoclonal against human RPL22L1 (1:50) overnight at 4°C in a moist chamber. The slides were then sequentially incubated with goat anti-rabbit IgG antibody-horseradish peroxidase conjugates for 30 min at 37°C, and 3′-3′ diaminobenzidine was used as the chromogen substrate. Finally, all slides were counterstained for nuclei with hematoxylin, dehydrated, and mounted. For the negative control, the primary antibody was replaced with normal rabbit IgG. RPL22L1 immunoreactivity in TMA samples was evaluated by three pathologists. The intensity of immunoreactivity on TMAs was graded on a scale from 0 to 3 based on a consensus of the three investigators.

Vectors
RPL22L1-ORF was PCR amplified and cloned into the pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA, USA). The siRNA (h) and control siRNA were purchased from RiboBio (Q000200916-1-B, Guangzhou, China). The luciferase reporter plasmid pGL4.17 was kindly
Transfection

All plasmids and siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Nude mouse tumor xenograft model

The animal experiments were approved by the Ethics Committee of Harbin Medical University (HMUIRB20150023) and performed according to the Guidelines of Laboratory Animal Usage of Harbin Medical University. Four-week-old BALB/c nude female mice were randomization assigned in each group, five mice per group. Each mouse was injected with $1 \times 10^6$ cells (in 200μl of phosphate-buffered saline [PBS]). Tumor growth was measured twice a week via bioluminescence imaging. Mice were injected intraperitoneally with 150μg / g D-luciferin (Bio-synth, Naperville, IL) in PBS and anesthetized with 2.5% isoflurane. Photons emitted from the mice were recorded by IVIS Lumina (Advanced Molecular Vision, Lincolnshire, UK) and presented as pseudo-color images overlaid on a gray-scale body image. All mice were euthanized by CO2 inhalation 30 days after injected. To ensure death following CO2 asphyxiation, cervical dislocation was performed.

Cell migration and invasion assays

Transwell cell migration and invasion assays were performed using Corning 8.0-mm Transwell inserts (8-μm pore size, 24-well plate) and BD BioCoat™ Matrigel™ Invasion Chambers (Corning Incorporated Life Sciences, Tewksbury, MA, USA) according to the manufacturer’s instructions.

Wound healing assay

The cell monolayer was scratched with a 10-μl pipette tip (in a 6-well plate). Photographs (magnification, ×40) were taken immediately and at each 24-h post-wounding until the wounds were obviously healed. For each assay, the experiments were performed at nine different positions and the whole assay was repeated three times.

Immunofluorescence staining

Cells were fixed in methanol and blocked for 1-h with 10% normal goat serum, 0.3% bovine serum albumin, 0.05% saponin, and 0.3% Triton X-100 in PBS. The primary antibodies were added and incubated at 4°C overnight. Cells were later washed with PBS and incubated with fluorescence-labeled secondary antibody. A fluorescence microscope (Leica) was used to capture the images.

Statistics

The RPL22L1 protein expression levels of TMAs were analyzed by Wilcoxon’s signed-rank tests. The $\chi^2$ test was used to examine associations between gene expression and clinical parameters. Other data were expressed as means ± SD, and the statistical significance of differences between two groups was evaluated using two-tailed independent Student’s $t$-tests. Statistical significance was declared if $P < 0.05$. Calculations were carried out using SPSS 13.0 (IBM; Armonk, NY, USA).
Results

*RPL22L1* was amplified via DMs and over-expressed in UACC-1598 cells

The OC cell line UACC-1598 contained amplified genes in the form of DMs. Oncogene amplification on DMs is representative of tumorigenesis, but does not occur in normal cells (Fig 1A). Using a FISH analysis, we detected amplified regions of *RPL22L1* that co-localized with *MYCN* on DMs (Fig 1B). Furthermore, we detected the amplification of *RPL22L1* in normal ovarian tissues, human lymphocytes, and UACC-1598 cells using PCR (Fig 1C). We also examined the amplification of *RPL22L1* in another three ovarian cancer cell lines (S1 Fig). RT-PCR confirmed the expression of *RPL22L1* in different samples (Fig 1D). These results indicated that *RPL22L1* was amplified via DMs in UACC-1598 cells.

**Fig 1.** *RPL22L1* was amplified on DMs and over-expressed in UACC-1598 cells. (A) Representative pictures of metaphase chromosomes of UACC-1598 and normal human lymphocytes. Arrows indicate DMs in UACC-1598 cells (magnification, ×1,000). (B) Representative images of FISH analysis. Metaphase chromosomes of UACC-1598 and human lymphocytes were detected with DNA probes. (a) Red probe indicates *RPL22L1* and (b) green probe indicates *MYCN*. (c) Both probes were located at the same locus on DMs as indicated by the arrows, (d) red probe indicates *RPL22L1* in normal human lymphocytes (magnification, ×1,000). (C) DNA amplification levels of *RPL22L1* detected by PCR. (D) RT-PCR result of mRNA expression levels of *RPL22L1* in different samples.

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DNA copy number and expression level of \textit{RPL22L1} in clinical OC samples

To determine the amplification of \textit{RPL22L1} in clinical, we used the Oncomine database (https://www.oncomine.org) to analyze the DNA copy number profiles of the \textit{RPL22L1} in a TCGA ovarian dataset (TCGA Ovarian 2, http://tcga-data.nci.nih.gov/tcga/) [20, 21]. This dataset clearly indicated a significant increase in the DNA copy number of \textit{RPL22L1} in OC compared to normal blood and ovarian tissues, threshold by $P < 10^{-4}$ (Fig 2A).

Further, we obtained publically available GEO expression datasets to analyze the expression of \textit{RPL22L1} in OC samples. Two GEO datasets based on the Affymetrix Human Genome U133 Plus 2.0 Array were used to evaluate \textit{RPL22L1} mRNA expression levels in OC and normal ovarian tissues. In each of the two datasets, \textit{RPL22L1} expression was significantly higher in OC than normal ovarian tissues (Fig 2B and 2C, $P < 0.05$, Student’s t-test).

To further examine the protein expression level of \textit{RPL22L1} in clinical specimens, four OC TMAs were used for IHC analyses. Staining intensity was estimated using an index of 0–3 in the cell nucleus and cytoplasm (S2 Fig). \textit{RPL22L1} was clearly expressed more highly in OC

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Fig 2. DNA copy number and expression level of \textit{RPL22L1} in clinical OC samples. (A) DNA copy number profiles of \textit{RPL22L1} in an ovarian serous cystadenocarcinoma data set registered in the Oncomine database (TCGA Ovarian 2 Dataset). Two independent microarray gene expression datasets (B) GSE29405 and (C) GSE27651 from GEO website were used to examine the expression level of \textit{RPL22L1} in OC. The raw.CEL files for the two databases were downloaded and normalized by RMA. \textit{RPL22L1} was more highly expressed in OC than in normal ovarian tissue (* $P < 0.05$, independent Student’s t-test). Representative pictures of \textit{RPL22L1} protein expression in (D) OC and (E) matched adjacent normal tissue by IHC (magnification, ×400).

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cytoplasm than in that of the adjacent normal tissues (Fig 2D and 2E) \((P = 0.008, \text{Wilcoxon's signed-rank test})\).

We examined the associations between RPL22L1 protein expression levels and clinicopathologic variables. RPL22L1 expression in cytoplasm of OC cells was strongly associated with stage, invasion, and lymph node metastasis \((P < 0.05, \text{Pearson's } \chi^2 \text{ test, Table 1})\). These results indicated that the expression of RPL22L1 is commonly higher in clinical OC specimens, and its expression level is associated with tumor progression, especially with invasion and lymph node metastasis.

RPL22L1 enhanced intraperitoneal xenograft tumor development \textit{in vivo}

To detect the role of high level RPL22L1 in tumor progression, we selected an OC cell line with low RPL22L1 expression, SKOV3, for further functional studies (Fig 3A). SKOV3 cells were stable transfection with luciferase previously and then stable transfection with RPL22L1 (Fig 3B and 3C). To explore the role of RPL22L1 in tumor progression \textit{in vivo}, we intraperitoneally injected SKOV3-RPL22L1 and control cells into nude mice. The xenografted tumors were measured using bioluminescence at the 30th day after injection, area of xenograft tumor in mice with SKOV3-RPL22L1 cells injected was larger than that of control cells injected (Fig 3D). The result suggested that high level of RPL22L1 contribute to tumor development.

RPL22L1 promoted OC cell migration and invasion

To confirm the role of RPL22L1 in OC cells, UACC-1598 cells were treated with three specific siRNAs against RPL22L1 (siRNA-1, siRNA-2, and siRNA-3). Since siRNA-2 exhibited the

| Variables              | Number | RPL22L1 expression | P value |
|------------------------|--------|--------------------|---------|
|                        |        | Low expression (0)  | High expression (1–3) |
| Age                    |        |                    |         |
| ≤ 50                   | 230    | 69 (30.0%)          | 161 (70.0%) | 0.102 |
| > 50                   | 200    | 46 (23.0%)          | 154 (77.0%) |
| Tumor differentiation  |        |                    |         |
| Well to moderate       | 177    | 62 (35.0%)          | 115 (65.0%) | 0.002* |
| Poor                   | 161    | 32 (19.9%)          | 129 (80.1%) |
| Stage                  |        |                    |         |
| 1                      | 211    | 75 (35.5%)          | 136 (64.5%) | 0.000* |
| 2–4                    | 219    | 40 (18.3%)          | 179 (81.7%) |
| TNM system             |        |                    |         |
| T                      |        |                    |         |
| T1                     | 214    | 76 (35.5%)          | 138 (64.5%) | 0.000* |
| T2–4                   | 216    | 39 (18.1%)          | 177 (81.9%) |
| N                      |        |                    |         |
| N0                     | 354    | 105 (29.7%)         | 249 (70.3%) | 0.003* |
| N1–3                   | 76     | 10 (13.2%)          | 66 (86.6%) |
| M                      |        |                    |         |
| M0                     | 383    | 108 (28.2%)         | 275 (71.8%) | 0.052 |
| M1                     | 47     | 7 (14.9%)           | 40 (85.1%) |

* \(P < 0.05\), all \(P\)-values were calculated for Pearson's \(\chi^2\) tests.

Table 1. Correlation between RPL22L1 expression and clinicopathological features in the cytoplasm of ovarian cancer (OC) tissues.

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most efficient knockdown of endogenous RPL22L1, it was chosen for subsequent analyses (S3 Fig). Except SKOV3-RPL22L1 and the control cells, we used another two OC cell lines HO-8910 and HO-8910PM with lower RPL22L1 expression to transient transfected with RPL22L1 for further functional study (S3 Fig). The effect of RPL22L1 on cell motility was characterized by wound-healing, transwell migration, and Matrigel invasion assays. Knockdown of RPL22L1 in UACC-1598 cells (1598-siRPL22L1) caused clear ression of cells migration and invasion. Over-expression of RPL22L1 in SKOV3-RPL22L1, HO-8910 (8910-RPL22L1), and HO-8910PM (8910PM-RPL22L1) cells could significantly enhance cells migration and invasion ($P < 0.05$, Fig 4). Cell growth rate was analyzed by an MTA assay, RPL22L1 had no influence on cell proliferation (data not shown). These results suggested that high level of RPL22L1 enhances OC cells migration and invasion.

**The expression level of RPL22L1 influences OC cell line EMT**

It has become increasingly clear that EMT is an integral component of the progression of epithelial-derived tumors [22, 23]. We found that SKOV3-RPL22L1 cells exhibited a spindle-shaped and fibroblastic morphology whereas 1598-siRPL22L1 cells exhibited shrinkage shapes and increased cell–cell adhesion (S4 Fig). Therefore, we examined whether RPL22L1 induced EMT could account for the RPL22L1-mediated changes in cells motility and invasion. Biochemical hallmarks of EMT include the loss of expression of epithelial marker proteins and concurrent increase in mesenchymal marker expression [22, 24]. Western blots and immunofluorescence analyses were used to evaluate the expression of epithelial and mesenchymal markers. After ectopic over-expression of RPL22L1 in SKOV3 cells, the expression of epithelial makers, such as E-cadherin, β-catenin, and α-catenin decreased, whereas the expression of vimentin, α-SMA, and fibronectin increased (Fig 5A and 5B). In 1598-siRPL22L1 cells, the expression levels of the mesenchymal markers vimentin and N-cadherin were degraded (Fig 5C). These results suggested that the expression level of RPL22L1 influences EMT in OC cells.
Discussion

DMs are malignant cytogenetic markers and are closely correlated with tumor progression [4]. Previously, we determined that RPL22L1 amplified on DMs, but its function is unclear. Many oncogenes are amplified via DMs in malignant tumor cells [34,38,39], such as EIF5A2 and MYCN, both of which are located at the same locus as RPL22L1 on DMs. We inferred that RPL22L1 may be involved in OC progression, and verified this in a series of in vitro and in vivo assays.

Using publically available TCGA and GEO expression datasets we found both DNA copy number and mRNA expression of RPL22L1 was significantly higher in OC tissues than in normal ovarian tissues. Protein expression of RPL22L1 was examined by IHC using four OC TMAs. We found that RPL22L1 expression was frequently higher in OC tissues compared with...
normal adjacent ovarian tissue ($P = 0.008$, Wilcoxon’s signed-rank test). Further, we found the expression level was significantly correlated with disease stage (81.7% in stage 2–4 versus 64.5% in stage 1) invasion depth (81.9% in T2–T4 versus 64.5% in T1), and lymph node metastasis (86.6% with versus 70.3% without metastasis), suggesting that the high level of RPL22L1 in OC cells may facilitate the invasive/metastatic phenotype. These findings underscore a potentially important role of RPL22L1 as an underlying biological mechanism in the development and progression of OC.

The results of the IHC analysis indicated that RPL22L1 may be involved in the pathogenesis of OC progression especially in metastasis. To confirm this hypothesis, nude mouse tumor xenograft, wound-healing, transwell migration, and Matrigel invasion assays were carried out to investigate the role of RPL22L1 in regulating OC cells motility, invasion. Over-expression of RPL22L1 obviously promoted tumor development in vivo and increased the migration and invasion ability of three OC cell lines in vitro. Moreover, the knockdown of endogenous RPL22L1 by siRNA in UACC-1598 cells inhibited migration and invasion in vitro. Migration and invasion are two important stages in tumor metastasis [25], and these functional assays strongly suggested that RPL22L1 plays an important role in promoting tumor metastasis via enhancing cell migration and invasion.

Many biological processes are associated with migration and invasion including EMT, a key event in tumor invasion and metastasis [26]. Over-expression of RPL22L1 reduced the expression of epithelial marker proteins (E-cadherin, β-catenin, and α-catenin) and increased the expression of mesenchymal markers (vimentin, α-SMA, and fibronectin), which are biochemical hallmarks of EMT [24, 27, 28]. However, after knockdown of RPL22L1 in UACC-1598 cells, the mesenchymal markers vimentin and N-cadherin were obviously down-regulated. EMT plays a critical role in promoting metastasis and during this process cells obtain migration and invasion capabilities, which promote metastasis [29, 30]. Accordingly, we inferred that the over-expression of RPL22L1 likely induced EMT to promote cell invasion and metastasis.

RPL22L1 is a paralog of RPL22, which is a RNA-binding protein component of the 60S ribosomal subunit. Ribosomal proteins are major components of ribosomes where cellular proteins are synthesized. To date, approximately 80 ribosomal proteins have been identified. In
addition to their key roles in protein synthesis, some ribosomal proteins are involved in extra-ribosomal functions, such as DNA repair, apoptosis, transcription regulation, and translation regulation [31–36]. An increasing number of reports have suggested that ribosomal proteins have oncogenic potential [37–39]. Recently, RPL22 has been found to be mutated or down-regulated in various cancers, including T-acute lymphoblastic leukemia, invasive breast carcinoma, and lung adenocarcinoma. Additionally, RPL22 directly represses the expression of RPL22L1 mRNA, and a lack of RPL22 causes a compensatory increase in RPL22L1 [40, 41]. These studies support our conjecture regarding the role of RPL22L1 in cancer progression.

Taken together, our results suggested that RPL22L1 plays an important role in OC progression by enhancing cell invasion and metastasis via inducing EMT. As RPL22L1 is a DM carried amplified gene, our data could help explain the function of DMs in cancer. RPL22L1 is a novel gene that is correlated with metastasis, and studies aiming to elucidate its function and modes of action may improve our understanding of the mechanisms underlying metastasis in OC.

**Supporting Information**

S1 Fig. DNA amplification levels of RPL22L1 detected by PCR. (TIF)

S2 Fig. Representative pictures of four different degrees of RPL22L1 staining (0–3) in both the nucleus and cytoplasm. (TIF)

S3 Fig. The efficient transfected in OC cell lines was detected by western blots. (TIF)

S4 Fig. Morphologies of cells were captured under a light microscope. (TIF)

S1 Text. Details of primary antibodies. (DOC)

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**Author Contributions**

Conceived and designed the experiments: YJ SF. Performed the experiments: NW JW YW YQ BP. Analyzed the data: NW CZ DS HS. Contributed reagents/materials/analysis tools: DT JB JG YY. Wrote the paper: NW JW KL WS XM.

**References**

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA: a cancer journal for clinicians. 2012; 62(1):10–29. doi: 10.3322/caac.20138 PMID: 22237781.

2. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000; 100(1):57–70. PMID: 10647931.

3. Jelovac D, Armstrong DK. Recent progress in the diagnosis and treatment of ovarian cancer. CA: a cancer journal for clinicians. 2011; 61(3):183–203. doi: 10.3322/caac.20113 PMID: 21521830; PubMed Central PMCID: PMC3576854.
4. Benner SE, Wahl GM, Von Hoff DD. Double minute chromosomes and homogeneously staining regions in tumors taken directly from patients versus in human tumor cell lines. Anti-cancer drugs. 1991; 2(1):11–25. PMID: 1720337.

5. Shimizu N. Molecular mechanisms of the origin of micronuclei from extrachromosomal elements. Mutagenesis. 2011; 26(1):119–23. doi: 10.1093/mutage/geq053 PMID: 21164192.

6. Shimizu N. Extrachromosomal double minutes and chromosomal homogeneously staining regions as probes for chromosome research. Cytogenetic and genome research. 2009; 124(3–4):312–26. doi: 10.1159/000218135 PMID: 19556783.

7. Von Hoff DD, McGill JR, Forseth BJ, Davidson KK, Bradley TP, Van Devanter DR, et al. Elimination of extrachromosomally amplified MYC genes from human tumor cells reduces their tumorigenicity. Proceedings of the National Academy of Sciences of the United States of America. 1992; 89(17):8165–9. PMID: 1518843; PubMed Central PMCID: PMC49877.

8. Ambros IM, Rumpler S, Luengmayr A, Strehl S, Kovar H, et al. Neuroblastoma cells can actively eliminate supernumerary MYCN gene copies by micronucleus formation—sign of tumour cell revertance? European journal of cancer. 1997; 33(12):2043–9. PMID: 9516850.

9. Canute GW, Longo SL, Longo JA, Shetler MM, Coyle TE, Winfield JA, et al. The hydroxyurea-induced loss of double-minute chromosomes containing amplified epidermal growth factor receptor genes reduces the tumorigenicity and growth of human glioblastoma multiforme. Neurosurgery. 1998; 42(3):609–16. PMID: 9526955.

10. Guan XY, Fung JM, Ma NF, Lau SH, Tai LS, Xie D, et al. Oncogenic role of eIF-5A2 in the development of ovarian cancer. Cancer research. 2004; 64(12):4197–200. doi: 10.1158/0008-5472.CAN-03-3747 PMID: 15205531.

11. Guan XY, Sham JS, Tang TC, Fang Y, Huo KK, Yang JM. Isolation of a novel candidate oncogene of ovarian cancer Journal international du cancer. 2011; 133(4):797–806. doi: 10.1002/ijc.23382041; PubMed Central PMCID: PMC3734650.

12. Ji W, Bian Z, Yu Y, Yuan C, Liu Y, Yu L, et al. Expulsion of micronuclei containing amplified genes contributes to a decrease in double minute chromosomes from malignant tumor cells. International journal of cancer International du cancer. 2014; 134(6):1279–88. doi: 10.1002/ijc.28467 PMID: 24027017; PubMed Central PMCID: PMC4233879.

13. Zhu J, Yu Y, Meng X, Fan Y, Zhang Y, Zhou C, et al. De novo-generated small palindromes are characteristic of amplicon boundary junction of double minutes. International journal of cancer Journal international du cancer. 2013; 133(4):797–806. doi: 10.1002/ijc.28084 PMID: 23382041; PubMed Central PMCID: PMC3734650.

14. Deng X, Zhang L, Zhang Y, Yan Y, Xu Z, Dong S, et al. Double minute chromosomes in mouse methotrexate-resistant cells studied by atomic force microscopy. Biochemical and biophysical research communications. 2006; 346(4):1226–33. doi: 10.1016/j.bbrc.2006.06.041 PMID: 16806082.

15. Yu L, Zhao Y, Quan C, Ji W, Zhu J, Huang Y, et al. Gemcitabine eliminates double minute chromosomes from human ovarian cancer cells. PloS one. 2013; 8(8):e71988. doi: 10.1371/journal.pone.0071988 PMID: 23991020; PubMed Central PMCID: PMC3750019.

16. Guan XY, Trent JM, Meltzer PS. Generation of band-specific painting probes from a single microdissected chromosome. Human molecular genetics. 1993; 2(8):1117–29. PMID: 806. doi:10.1093/jcp.21223 PMID: 23382041; PubMed Central PMCID: PMC3130734.

17. King ER, Tung CS, Tsang YT, Zu Z, Lok GT, Deavers MT, et al. The anterior gradient homolog 3 (AGR3) gene is associated with differentiation and survival in ovarian cancer. The American journal of surgical pathology. 2011; 35(6):904–12. doi: 10.1097/PAS.0b013e31821212ae22 PMID: 21451962; PubMed Central PMCID: PMC3095702.

18. Stany MP, Vathipadiesek V, Ozbun L, Stone RL, Mok SC, Xue H, et al. Identification of novel therapeutic targets in microdissected clear cell ovarian cancers. PloS one. 2011; 6(7):e21121. doi: 10.1371/journal.pone.0021121 PMID: 21754983; PubMed Central PMCID: PMC3130734.

19. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, S切尔 F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003; 4 (2):249–64. doi: 10.1093/biostatistics/4.2.249 PMID: 12925520.

20. Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB, et al. Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. Neoplasia. 2007; 9(2):166–80. PMID: 17356713; PubMed Central PMCID: PMC1813932.

21. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, et al. ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia. 2004; 6(1):1–6. PMID: 15068665; PubMed Central PMCID: PMC1635162.

22. Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, et al. Epithelial—mesenchymal and mesenchymal—epithelial transitions in carcinoma progression. Journal of cellular physiology. 2007; 213(2):374–83. doi: 10.1002/jcp.21223 PMID: 17680632.
23. Gos M, Miloszewska J, Przybyszewska M. [Epithelial-mesenchymal transition in cancer progression]. Postepy biochemii. 2009; 55(2):121–8. PMID: 19824467.

24. Tomaskovic-Crook E, Thompson EW, Thiery JP. Epithelial to mesenchymal transition and breast cancer. Breast cancer research: BCR. 2009; 11(6):213. doi: 10.1186/bcr2416 PMID: 19909494; PubMed Central PMCID: PMC2815537.

25. Bravo-Cordero JJ, Hodgson L, Condeelis J. Directed cell invasion and migration during metastasis. Current opinion in cell biology. 2012; 24(2):277–83. doi: 10.1016/j.ceb.2011.12.004 PMID: 22209238; PubMed Central PMCID: PMC3320684.

26. Kang Y, Massague J. Epithelial-mesenchymal transitions: twist in development and metastasis. Cell. 2004; 118(3):277–9. doi: 10.1016/j.cell.2004.07.011 PMID: 15294153.

27. Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. Science signaling. 2014; 7(344):re8. doi: 10.1126/scisignal.2005189 PMID: 25249658; PubMed Central PMCID: PMC4372086.

28. Bronsert P, Enderle-Ammour K, Bader M, Timme S, Kuehs M, Csanadi A, et al. Cancer cell invasion and EMT marker expression: a three-dimensional study of the human cancer-host interface. The Journal of pathology. 2014; 234(3):410–22. doi: 10.1002/path.4416 PMID: 25081610.

29. Tsai JH, Yang J. Epithelial-mesenchymal plasticity in carcinoma metastasis. Genes & development. 2013; 27(20):2192–206. doi: 10.1101/gad.225334.113 PMID: 24142872; PubMed Central PMCID: PMC3320684.

30. Luo M, Brooks M, Wicha MS. Epithelial-mesenchymal plasticity of breast cancer stem cells: implications for metastasis and therapeutic resistance. Current pharmaceutical design. 2015; 21(10):1301–10. PMID: 25506895.

31. Zimmermann RA. The double life of ribosomal proteins. Cell. 2003; 115(2):130–2. PMID: 14567909.

32. Mazumder B, Sampath P, Seshadri V, Maitra RK, DiCorleto PE, Fox PL. Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. Cell. 2003; 115(2):187–98. PMID: 14567916.

33. Chavez-Rios R, Arias-Romero LE, Almaraz-Barrera Mde J, Hernandez-Rivas R, Guillen N, Vargas M. L10 ribosomal protein from Entamoeba histolytica share structural and functional homologies with QM/Jif-1: proteins with extraribosomal functions. Molecular and biochemical parasitology. 2003; 127(2):151–60. PMID: 12672524.

34. Neumann F, Krawinkel U. Constitutive expression of human ribosomal protein L7 arrests the cell cycle in G1 and induces apoptosis in Jurkat T-lymphoma cells. Experimental cell research. 1997; 230(2):252–61. PMID: 9024784.

35. Khanna N, Reddy VG, Tuteja N, Singh N. Differential gene expression in apoptosis: identification of ribosomal protein S29 as an apoptotic inducer. Biochemical and biophysical research communications. 2000; 277(2):476–86. doi: 10.1006/bbrc.2000.3688 PMID: 11032747.

36. Naora H, Takai I, Adachi M, Naora H. Altered cellular responses by varying expression of a ribosomal protein gene: sequential coordination of enhancement and repression of ribosomal protein S3a gene expression induces apoptosis. The Journal of cell biology. 1998; 141(3):741–53. PMID: 9566973; PubMed Central PMCID: PMC2132756.

37. Liu JM, Ellis SR. Ribosomes and marrow failure: coincidental association or molecular paradigm? Blood. 2006; 107(12):4583–8. doi: 10.1182/blood-2005-12-4831 PMID: 16507776.

38. Narla A, Ebert BL. Ribosomopathies: human disorders of ribosome dysfunction. Blood. 2010; 115(16):3196–205. doi: 10.1182/blood-2009-10-178129 PMID: 20194897; PubMed Central PMCID: PMC2858486.

39. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D. Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. Human mutation. 2007; 28(12):1178–82. doi: 10.1002/humu.20608 PMID: 17647292.

40. Zhang Y, Duc AC, Rao S, Sun XL, Billbee AN, Rhodes M, et al. Control of hematopoietic stem cell emergence by antagonistic functions of ribosomal protein paralogs. Developmental cell. 2013; 24(4):411–25. doi: 10.1016/j.devcel.2013.01.018 PMID: 23449473; PubMed Central PMCID: PMC3586312.

41. O’Leary MN, Schreiber KH, Zhang Y, Duc AC, Rao S, Hale JS, et al. The ribosomal protein Rpl22 controls ribosome composition by directly repressing expression of its own paralog, Rpl22l1. PLoS genetics. 2013; 9(8):e1003708. doi: 10.1371/journal.pgen.1003708 PMID: 23990801; PubMed Central PMCID: PMC3750023.