Original Article
ADP-ribosylation factor-like 4C (ARL4C), a novel ovarian cancer metastasis suppressor, identified by integrated genomics

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Abstract: Understanding the molecular mechanisms involving the initiation, progression, and metastasis of ovarian cancer is important for the prevention, detection, and treatment of ovarian cancer. In this study, two ovarian cancer cell lines, HO-8910 and its derivative HO-8910PM with highly metastatic potential, were applied to comparative genomic hybridization (CGH) analysis. We found 14 chromosome fragments with different copy numbers between the two cell lines, one (2q36.1-37.3) of which was confirmed to be one-copy loss in HO-8910PM by fluorescent in situ hybridization (FISH). Using the microarray data on gene expression profiles from these cell lines, 6 significantly expression-decreased genes located on 2q36.1-37.3 in HO-8910PM were identified. Of the 6 genes, ARL4C was identified as a novel ovarian cancer-related gene using integrated molecular and genomic analyses. ARL4C mRNA expression was validated by quantitative PCR to be markedly decreased in HO-8910PM cells, compared to that in HO-8910. Both overexpression and knockdown of ARL4C demonstrated that low ARL4C expression promotes the migration but not influences proliferation capability of ovarian cancer cells in vitro, indicating its specific role in ovarian cancer progression. Furthermore, ovarian cancer patients with medium and high expression of ARL4C mRNA had a favorable prognosis compared to those with low expression, suggesting the ARL4C could be a potential predictor for ovarian cancer prognosis.

Keywords: Ovarian cancer, genomics, metastasis, ARL4C, tumor cell biology, prognosis

Introduction

Ovarian cancer is the fourth leading cause of cancer mortality among women in Western societies [1]. There were 22,280 new cases of ovarian cancer diagnosed in the US and 15,500 deaths in 2012 [2]. Due to the lack of specific symptoms and signs at early stage of the disease onset, most of the patients with ovarian cancer are diagnosed late; about 75% of patients have extensive metastasis in the abdominal cavity when diagnosed. Patients with ovarian cancer have less than 50% chance of 5-year survival. A majority of patients die of disease recurrence or metastasis, even though the tumors are sensitive to initial treatment and the patients are cleared from the primary malignancy [3, 4]. Direct spread and peritoneal seeding (exfoliation of cells into the peritoneal cavity) are the main routes of ovarian cancer metastasis. Tumor cells directly invade adjacent organs, and widely seed on the peritoneal and omental surfaces, causing massive ascites. Hematogenous metastasis occurs at advanced stage, and the most common sites are liver, lung, pleura, kidney, bone, adrenal gland and spleen [5].

Recent studies have revealed many novel metastasis-related genes in ovarian cancer. Scaffolding adaptor protein Gab2, over-expressed in ovarian cancer cells, inhibits E-cadherin expression and promotes characteristics of EMT in ovarian cancer cells by activation
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of the PI3K-Zeb1 pathway [6]. Mucin 4 (MUC4) induces EMT through upregulation of N-cadherin and promotes metastasis of ovarian cancer cells [7]. Lysoosphatidic acid (LPA)-induced Rac activation is a prerequisite for ovarian cancer metastasis, and the integrity of SOS1/EPS8/ABI1 tri-complex is a determinant of ovarian cancer metastasis [8]. In addition, the latest study indicates that adipocytes, a major component of tumor microenvironment, provide fatty acids as energy for rapid growth of tumor cells and promote migration and invasion of ovarian cancer cells to the omentum through their secreted adipokines, such as interleukin-8 (IL-8). Fatty acid binding protein (FABP4), a transport coordinator of lipids between adipocytes and tumor cells, plays a key role in ovarian cancer metastasis [9]. Although recent studies have revealed several molecular mechanisms involved in the metastasis of ovarian cancer, much remains unknown regarding how cancerous cells initiate metastasis and colonize at a secondary organ. So, more studies are needed to further explore the mechanisms of ovarian cancer metastasis.

Genetic alterations are the key events in the initiation and promotion of many malignant tumors, including ovarian cancer [10, 11]. Usually, cancer-related genetic changes can result in activation of oncogenes and inactivation of tumor suppressor genes, which are responsible for tumor metastasis, drug resistance and other biological behaviors of malignant tumor cells [12, 13]. Comparative genomic hybridization (CGH) is an effective method to identify large changes in genomic DNA, such as copy number variation, and has been shown to be useful in identification of novel oncogenes and tumor suppressor genes with accurate chromosomal location [13]. In the present study, using a CGH chip combined with gene expression profiling, we compared ovarian cancer cells with high (HO-8910PM) and low (HO-8910) metastatic potential, and identified ARL4C, located on chromosome 2q37.1, to be down-regulated in HO-8910PM cells, as compared with HO-8910. To further explore the biological actions of ARL4C in ovarian cancer, we investigated the effects of ARL4C over- and down-expression mediated by lentiviral vectors on colony formation, cell proliferation and motility of ovarian cancer cells. In addition, we analyzed ARL4C mRNA expression in association with clinico-pathological features of ovarian cancer and patient survival.

Materials and methods

Cell lines and culture

Ovarian cancer cell lines, HO-8910 and HO-8910PM, were established from our previous studies [14, 15]; SKOV3, OVCAR3 and Es-2 cells were purchased from American Type Culture Collection (Manassas, VA). A2780 cells were obtained from Sigma-Aldrich Company (St Louis, MO). COC1 cells were bought from 3D High Throughput Screening Co., Ltd (Shanghai, China). OVCAR8 cells were a gift from Dr. Qiaojun He (Zhejiang University, Zhejiang, China). SKOV3 cells were cultured in DMEM medium, and HO-8910, HO-8910PM, A2780, SKOV3, OVCAR3, OVCAR8, COC1, and Es-2 cells were cultured in RPMI-1640 medium containing 10% newborn bovine serum, supplemented with 100 U/ml penicillin and 125 µg/ml streptomycin. All cell cultures were incubated at 37°C with 5% CO₂.

DNA isolation and CGH analysis

DNA was isolated from cells using the standard phenol/chloroform method. The Affymetrix GeneChip® Mapping Assay, in conjunction with the GeneChip Human Mapping 10K Array 2.0 (Affymetrix Inc., Santa Clara, CA), were used to analyze chromosomal regions with different copy numbers between HO-8910 and HO-8910PM cell lines. The analysis was performed according to the assay manual. The protocol started with 250 ng of genomic DNA which was first digested with 20,000 U/ml Xba I restriction enzyme (New England Biolab Ltd, HERTS, UK) and then ligated with a special sequence using T4 DNA Ligase (New England Biolab Ltd, HERTS, UK). Following the ligation, PCR procedure was performed to amplify the ligated DNA, and then followed by fragmentation and end-labeling of PCR products. The labeled DNA was hybridized to the GeneChip array. After hybridization, the array was washed, stained, scanned and read. Chromosome Copy Number Analysis Tool (CN-AT) software 4.0 (Affymetrix Inc., Santa Clara, CA) was used to analyze the chromosomal copy number changes. Data were normalized with quartile normalization and log2 ratio; replicated data points that exceeded a standard deviation of 0.075 were excluded. Paired copy number (CN) analysis with Hidden Markov Model (HMM) parameters was applied, and DNA from HO-8910 cells was used as reference.
Fluorescent in situ hybridization (FISH)

Chromosomes from HO-8910 and HO-8910PM ovarian cancer cells were prepared with colchicine at a final concentration of 0.07 μg/ml. Four biotin labeled bacterial artificial chromosome (BAC) clone probes separately mapping onto 2q35, and 2q37.1 were purchased from Si-noGenoMax Research Center Co., Ltd (Beijing, China). These probes were used to separately hybridize to the preparations of fixed cell nuclei and metaphases previously dehydrated and denatured for 2 minutes in 70% formamide at 72°C. The probe was denatured for 5 minutes at 72°C, and the hybridization was performed at 37°C overnight. After washing in 0.4 × SSC at 72°C for 2 minutes and 2 × SSC at room temperature for 30 seconds, avidin-FITC was added to enlarge the signal of hybridization for 40 minutes at 37°C, followed by antiavidin incubation for 40 minutes at 37°C. After washing in 2 × SSC at room temperature, PI was added on the slide. Hybridization signal was observed under a fluorescence microscope (Nikon, Tokyo, Japan) with FITC filter and photographed at a magnification of 400 ×. More than 30 metaphase and interphase cells were analyzed for each sample. Chromosomes from normal peripheral blood lymphocytes were used as control. Bright and round green dots located on the sister chromatid were determined as real signals.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

RNeasy Mini kits (QIAGEN Inc., Valencia, CA) were used to extract total RNA from cells and tissue. RNA (500 ng) was reverse transcribed to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA). Quantitative PCR was performed to determine the expression of ARL4C mRNA in each tumor sample, using GAPDH as an endogenous control for calibration. The primer sequences were designed using an online tool (http://www.idtdna.com), and the primers were ordered from Invitrogen Corp (Shanghai, China). The primer sequences were: TGG AAG GCT CAG TTG TCG GAA AGA (ARL4C forward), TAC ACA TGG ACA GGG TCC AAA CCA (ARL4C reverse); GAA GGT GAA GGT CGG AGT C (GAPDH forward), and GAA GAT GGT GAT GGG ATT TC (GAPDH reverse). ABI 7500 real-time PCR system (Applied Biosystems Inc., Foster City, CA) was used for qPCR reaction. The PCR solution (25 μl) contained 5 ng/μl cDNA, 12.5 μl Power SYBR Green PCR Master Mix (Bioer Technology Inc., Hangzhou, China) and a pair of primers at a final concentration of 0.1 μM for ARL4C and GAPDH. Dissociation curve analysis was performed after PCR amplification to confirm the size of PCR products. All tumor samples were analyzed in duplicate along with negative controls. Real-time PCR results were recorded as Ct values (threshold cycle). To adjust for the total amount of cDNA used for analysis in each sample, a ΔCt was calculated based on the difference in Ct values between the target ARL4C and housekeeping gene GAPDH. ΔCt was further converted to an expression index (EI) based on the formula 2^{-ΔCt}.

Western blot analysis

Cultured cells were harvested with 0.02% EDTA and 0.025% trypsin, rinsed three times in phosphate-buffered saline (PBS), and lysed in 300 μl SDS-lysis buffer supplemented with a cocktail of inhibitors for protease. Protein concentration in cell lysate was measured with the Bradford colorimetric assay (Bio-Rad, Richmond, California). Thirty μg of total protein from each sample were electrophoresed on an 8% SDS-PAGE gel, and the proteins on the gel were transferred onto a PVDF membrane (Millipore, MA). The membranes were blocked with TBST containing 5% non-fat milk for one hour at room temperature, and then incubated with anti-ARL4C antibody (Atlas Antibodies company, Stockholm, Sweden) for one hour at room temperature, following by further incubation with an HRP-conjugated secondary antibody and detection with ECL regent (Millipore, MA). GAPDH (Sigma, St Louis, MO) was used as a loading control.

ARL4C knockdown and overexpression

The lentiviral vector with shRNA for ARL4C, pLenti-U6-tdTomato-puromycin-shRNA-ARL4C vector, which contains a red fluorescent protein (tdTomato) marker for cell tracking, and the lentiviral vectors carrying ARL4C, pLenti-EF1α-copGFP-puromycin-ARL4C cDNA expression lentiviral Vector, which contains a green fluorescent protein (copGFP) marker, were purchased from 3D High Throughput Screening Co., Ltd (Shanghai, China). Empty lentiviral vector was used as control. The lentiviral vectors and pHelper plasmids were co-transfected into
The study analyzed ARL4C mRNA expression in fresh frozen tumor samples of 212 patients who underwent surgery for primary ovarian cancer. The patients were divided into two groups: those with high ARL4C expression and those with low ARL4C expression. The ARL4C expression levels were determined by quantitative real-time PCR (qRT-PCR) using specific primers for ARL4C. The results showed a significant difference in ARL4C expression between the two groups, with higher expression in the high ARL4C group compared to the low ARL4C group. The study also found a correlation between high ARL4C expression and worse clinical outcomes, such as shorter overall survival and progression-free survival. These findings suggest that ARL4C may be a potential biomarker for ovarian cancer and could be used in clinical settings to predict patient outcomes.
cancer in the Department of Gynecology and Obstetrics at University of Turin in Italy. The specimens were collected for a clinical study of epithelial ovarian cancer that was approved by the university’s ethical review committee. The average age of patients at surgery was 57.6 years (SD, 11.5; range, 26-82). Follow-up information was available for 203 patients who were followed from surgery to June 2001. The overall follow-up time ranged from 0.6 to 114 months, and the median was 31 months. The median disease progression-free survival was 20.6 months. Disease stage was classified according to the criteria of FIGO (the International Federation of Gynecologists and Obstetricians) [16]. Of the 211 patients with information on disease stage, 51 (24.2%) were diagnosed with stage I disease, 12 (5.7%) were stage II, 133 (63.0%) were stage III, and 15 (7.1%) were stage IV. Histological type determined following the World Health Organization (WHO) criteria [17] included serous, endometrioid, mucinous, clear cell, and other epithelial tumors. For data analysis, tumor histotype were grouped into non-serous (59.9%, n=127) and serous (40.1%, n=85). Of the 211 patients with information on tumor grade, most patients (65.4%, n=138) had grade 3 tumors (poorly differentiated); few had grade 1 or 2 (34.6%, n=73). Most of the patients received standard postoperative chemotherapy after cytoreduction surgery. Of the patients treated, 90 (42.4%) had no residual tumor, but 116 (54.7%) had residual tumors. Six patients lacked the information on residual tumor. Patient’s response to chemotherapy was evaluated one month after the last cycle of treatment through clinical examination, imaging, and serum CA125. For measurable disease, treatment response was assessed following the WHO criteria [17]. For non-measurable disease, progression was defined by CA125, doubling from the upper limit of normal range [18]. For data analysis, PR (partial response), SD (stable disease), and PD (progressive disease) were grouped together as poor responders to compare with CR (complete response). Of the 212 patients, 68.7% (n=145) had complete response to treatment, and 25.9% (n=55) had poor response. Twelve patients (5.4%) had no information on treatment response.

Statistical analysis

Data from in vitro experiments were expressed as means and standard deviations (SD) for 3 independent experiments using three different preparations. The differences between means
were compared using the Student’s t-test. For patient data, ARL4C mRNA expression was analyzed both as a continuous and categorical variable. For categorical analysis, the expression data were grouped into low, middle and high categories based on tertile distributions. Associations between ARL4C expression and clinical variables were analyzed using the Chi-square test. Survival analysis was performed for progression-free and overall survival using the Cox proportional hazards regression and Kaplan-Meier curves. All p-values were two-sided, and a probability of 0.05 or smaller was considered statistically significant. SPSS version 11.0 (SPSS Inc., Chicago, IL) was used for data analysis.

**Results**

**Comparison of chromosome fragments between cell lines**

HO-8910PM had 14 chromosome fragments with different copy numbers from HO-8910 cell line, mainly in chromosomes 1, 2, 3, 5, 6, 9, 11, 15, 20 and X. 85.7% (12/14) of the fragments lost one copy, and 14.3% (2/14) of the fragments gained one (Table 1). The shortest fragment (0.108 kb) was located on chromosome X p21.3, and the longest one (29641.546 kb) was on chromosome 5q21.3-31.2. Chromosome 2q36.1-37.3 was the second longest fragment (19020.56 kb) which had only one copy in HO-8910PM cells.

To validate the results of CGH analysis, we performed FISH assay on these two cell lines using one commercially available biotin-labeled BAC probes to detect the copy number change of chromosome fragments in 2q37.1. Consistent with the CGH results, two chromosomal hybridization signals were found for 2p37.1 in HO-8910, but only one was shown in HO-8910PM, suggesting a hemizygous deletion of chromosome 2p37.1 in the latter cells (Figure 1).

**Analysis of gene expression in ovarian cancer cells**

Based on the results of our CGH and FISH analyses, we focused our genetic evaluation on chromosome 2q36.1-37.3. Using our previous gene-expression profiling data [19], we found that 6 genes located on chromosome 2q36.1-

<table>
<thead>
<tr>
<th>Chromosome Location</th>
<th>Locus</th>
<th>Gene</th>
<th>Signal Log Ratio (HO-8910PM vs H08910)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr2q37.1</td>
<td>NM_005737</td>
<td>ARL4C</td>
<td>-3.8</td>
</tr>
<tr>
<td>chr2q37.3</td>
<td>NM_023083.3</td>
<td>CAPN10</td>
<td>-3.8</td>
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<td>chr2qter</td>
<td>NM_001463.3</td>
<td>FRZB</td>
<td>-3.4</td>
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<td>chr2q37</td>
<td>NM_001172416.1</td>
<td>KCNJ13</td>
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<td>SP100</td>
<td>-2.9</td>
</tr>
<tr>
<td>chr2q37.3</td>
<td>NM_001244008.1</td>
<td>KIF1A</td>
<td>-2.7</td>
</tr>
</tbody>
</table>
ARL4C in ovarian cancer

37.3 were down-regulated (Signal Log Ratio [SLR] ≤ -2.7) in HO-8910PM, compared to HO-8910. ARL4C, which encodes ADP-ribosylation factor-like 4C, was the most obviously differentiated gene with 3.8-fold decreased expression in HO-8910PM than in HO-8910 (Table 2). The other five genes, CAPN10, FRZB, KCN13, SP100 and KIF1A, were found to be gain or loss on copy number in ovarian cancer tissues according to the COSMIC CNV data (Figure 2A), whereas ARL4C was annotated to be rather deleted or decreased in mRNA expression than amplified in several tumors, such as glioma, prostate cancer, and cervical cancer by cbioPortal data (Figure 2B), which is in line with our observation that ARL4C deletion in ovarian cancer cell line might have more metastatic potential. Hence, we concentrated on the correlation between ARL4C CNV/expression with the metastatic potential in the subsequent studies.

And then, ARL4C protein and mRNA expression was analyzed in HO-8910, HO-8910PM, A2780, OVCAR3, SKOV3, OVCAR8, COC1, and Es-2 cells by Western blot and qPCR. ARL4C protein expression was either low or depleted in HO-8910, HO-8910PM, A2780 and OVCAR3, compared to that in SKOV3, OVCAR8, COC1 and Es-2 (Figure 3A). The protein level of ARL4C was too low to be investigated in HO-8910 by Western blot, however, the mRNA expression of ARL4C in HO-8910PM was significantly decreased by 30% (p=0.004), compared to that in HO-8910 (Figure 3B). This result is consistent with our CGH and FISH data.

ARL4C overexpression or knockdown influences migration but not proliferation capability of ovarian cancer cells in vitro

To evaluate the effects of ARL4C on the behaviors of ovarian cancer cells, we performed lentiviral vector-mediated overexpression of ARL4C in ARL4C-low expressed HO-8910PM and OVCAR3 cell lines and knockdown of ARL4C in ARL4C-highly expressed SKOV3 cell line. As shown in Figure 4A-C, 72 hours after transfection, 80% cells were observed with fluorescence (expression vector in green and shRNA vector in red). ARL4C overexpression and knockdown were validated by Western blot, respectively (the lower panel of Figure 4A-C). For ARL4C knockdown in SKOV3 cells, two shRNA species were applied to rule out the possibility of off-target effects in RNAi experiments [20]. The results showed that the ARL4C was totally silenced on protein level in SKOV3 cells following the treatment of the two shRNA species, indicating that the knockdown effects were not caused by off-target effects.

And then, the effect of ARL4C expression on ovarian cancer cell growth was investigated by

Figure 3. ARL4C protein and mRNA expression in ovarian cancer cell lines. A. ARL4C protein levels examined by western blot. GAPDH was applied for input control. B. ARL4C mRNA expression examined by qPCR. ARL4C mRNA expression was calculated relative to that in HO-8910 cell line.
colony formation and MTS assay. As shown in Figure 5, neither ARL4C overexpression nor ARL4C lower expression had obvious effects on colony formation (Figure 5A, 5B) and proliferation (Figure 5C) of ovarian cancer cells.

Subsequently, transwell migration experiments showed that the migration capability of ARL4C-overexpressed HO-8910-PM and OVCAR3 cells were decreased to 20% or so of the control cells, (Figure 6A, 6B, p < 0.001 and p < 0.01 for HO-8910-PM and OVCAR3 cells, respectively). Conversely, ARL4C knockdown increased the migration ability of SKOV3 cells by more than 70% when compared to the control cell (Figure 6C, p < 0.001 for the two shARL4C species).

These results suggested that ARL4C should suppress the migration of ovarian cancer cells in vitro.

Associations of ARL4C mRNA expression with disease characteristics and patient survival

We then examined possibility that the ARL4C expression is associated with the clinical outcome of patients with ovarian tumor. ARL4C expression was detected in the tumor samples, and the expression ranged from < 0.004 to 3.930 E1 with a median of 0.013 E1. No significant associations were found between ARL4C expression and disease stage, tumor grade, histological type, or residual tumor size.
However, there was a trend that ARL4C expression was lower in patients with residual tumors or who had poor treatment response than in those without residual tumors or who had good treatment response (0.009 versus 0.019, p=0.095, and 0.008 versus 0.014, p=0.055), respectively (Table 3).

ARL4C expression was not associated with disease progression-free survival, but there was a significant association between ARL4C and overall survival of ovarian cancer patients. Patients having medium or high ARL4C expression had better survival time than those with low expression. The 5-year overall survival rate in patients with low expression of ARL4C was 35%; while for patients with medium and high expression of ARL4C, the rate was 52%. The medium survival times for these two groups were 38.4 and 64.3 months, respectively (Figure 7). Cox regression analysis showed that high ARL4C expression was associated with lower risk of death and the association was independent of other prognostic factors for ovarian cancer. Patients with medium or high levels of ARL4C mRNA had hazard ratios of
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0.59 (95% CI: 0.39-0.91; P=0.017), after adjusting for age at surgery, disease stage, tumor grade, histological type and residual tumor size (Table 4).

Discussion

In the present study, we demonstrated that the genomic profile of a highly metastatic cell line HO-8910PM was different from that of its parental cell line HO-8910, with the former displaying fewer copy numbers in several chromosome fragments. Using FISH, we further confirmed that HO-8910PM had one copy number less than HO-8910 in chromosome fragment 2q36.1-37.3.

And then, comparison of gene expression profiles between the two cell lines indicated that 6 genes located on chromosome 2q36.1-37.3 had lower expression in HO-8910PM than in HO-8910. ARL4C was the most obviously differentiated gene with 3.8-fold decreased expression in HO-8910PM than in HO-8910. Moreover, the other 5 genes were reported to be amplified or deleted in ovarian cancer tissues by COSMIC database (Figure 2A), suggesting that these genes might not be suitable candidates for further studies. Therefore, ARL4C, the only one gene reported to be deleted in various cancers by cBioPortal data (Figure 2B), was chosen for further functional experiments.

Figure 6. ARL4C overexpression or knockdown significantly influences ovarian cancer cells migration in vitro. Ovarian cancer cells were infected by empty vector lentivirus (control), ARL4C overexpression or ARL4C shRNA lentivirus. 72 hours later, infected cells were seeded into the upper chamber. 24 hours later, the migrated cells were stained with crystal violet and photographed. Magnification: 200×. Error bars indicated SD from measurement of triplicate experiments. **, P<0.01; ***, P<0.001. A. ARL4C overexpression significantly suppressed migration of HO-8910PM cells. B. ARL4C overexpression significantly suppressed migration of OVCAR3 cells. C. ARL4C knockdown significantly promoted migration of SKOV3 cells.
ADP-ribosylation factor-like 4C (ARL4C), also known as ARL7, is a member of the ADP-ribosylation factor family of GTP-binding proteins [21, 22]. In 1999 Jacobs et al. isolated cDNA containing a full-length coding sequence of ARL4C from human bladder epithelia. The ARL4C protein has 192 amino acids, of which 71% were in homology with mouse ARL4, 59% in homology with human ARL6 and 49% in homology with ARF1 [23]. ARL4C is closely similar to ARL4A and ARL4D, and each has a nuclear localization signal and an unusually high guanine nucleotide exchange rate [23-25]. Early studies found that ARL4C has an inherently fast...

Table 3. Association of ARL4C mRNA expression with clinical and pathological characteristics of ovarian cancer

<table>
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<tr>
<th>Variable</th>
<th>n</th>
<th>Median (5th-95th)</th>
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<td>I-II</td>
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<td>0.008 (0-0.859)</td>
<td></td>
<td>24 (43.6)</td>
<td>15 (27.3)</td>
<td>16 (29.1)</td>
<td></td>
</tr>
</tbody>
</table>

*Expression levels at the 5th and 95th percentiles; Wilcoxon two-sample test with t approximation; Low: Expression Index (EI) <0.004; Middle: Expression Index (EI) 0.005-0.047; High: Expression Index (EI) > 0.054-3.930; p-value from Chi-square test.

Figure 7. Kaplan-Meier curves for overall survival of patients with ovarian cancer according to ARL4C mRNA levels.
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GTP enzyme activity and plays an important role in apolipoprotein A1 dependent cholesterol secretion process [26]. Recent studies found that ARL4C interacts with alpha-tubulin and modulates intracellular vesicular transport [27]. However, there are no reports that ARL4C is involved in tumor initiation and progression yet.

We then assessed the protein and mRNA expression of ARL4C in 8 ovarian cancer cell lines, including HO-8910 and HO-8910PM. Unfortunately, the protein level of ARL4C in HO-8910 was too low to be investigated by western blot, hence the protein levels between HO-8910 and HO-8910PM could not be compared. However, ARL4C mRNA expression was significantly decreased in HO-8910PM compared to that in HO-8910, supporting that ARL4C deletion in HO-8910PM leads to functional impairment on mRNA, and perhaps protein levels.

Subsequently, our in vitro functional experiments demonstrated that ARL4C negatively regulate motility of ovarian cancer cell. Considering that the suppression effects on migration were observed consistently in 3 ovarian cancer cell lines (HO-8910PM, OVCAR3 and SKOV3), it is not likely that this inhibition was caused by artificial events. Additionally, ARL4C expression has no effect on growth of ovarian cancer cells, proposing that ARL4C may be not a typical tumor suppressor gene, i.e., it regulates migration but not growth of cancer cells. Thus, the genomic variations on ARL4C may be evolutionally late events in tumor progression. Recently, Matsumoto et al reported that Wnt and growth factor signaling induces Arl4c expression [28]. The Wnt/β-catenin pathway is thought to be one of the major signaling pathways involved in epithelial-to-mesenchymal transition (EMT), a process occurred in the initiation of metastasis for cancer progression [29, 30]. The mechanism by which ARL4C regulates cancer cell migration might lie in the crosstalk between ARL4C and EMT process, which warrants further studies in more cancer cell lines and tissue samples.

Furthermore, our analysis of patient tumor samples provided additional evidence in support of our in vitro findings. ARL4C mRNA expression in tumor samples tended to be lower in patients with residual tumors or poor treatment response than in those without residual tumors or good treatment response. In addition, ARL4C mRNA expression was also positively associated with overall survival time. Patients with high or medium levels of expression had longer overall survival than those with low expression. These findings suggest that ARL4C may play a role in ovarian cancer progression and could be a candidate marker for ovarian cancer prognosis.

Table 4. Associations of ARL4C mRNA expression and patient survival from Italy

<table>
<thead>
<tr>
<th></th>
<th>Crude HR</th>
<th>95% CI</th>
<th>p-value</th>
<th>Adjust HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease-Free Survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low ARL4C</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium and High ARL4C</td>
<td>0.85</td>
<td>0.55-1.31</td>
<td>0.464</td>
<td>0.82</td>
<td>0.53-1.28</td>
<td>0.387</td>
</tr>
<tr>
<td>Overall Survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low ARL4C</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium and High ARL4C</td>
<td>0.61</td>
<td>0.40-0.93</td>
<td>0.021</td>
<td>0.59</td>
<td>0.39-0.91</td>
<td>0.017</td>
</tr>
</tbody>
</table>

1 H: Hazard Ratios from Cox models were adjusted by grade, histology, stage and residual tumor. Significance level of p=0.05. 2 Wald confidence limits. 3 Hazard ratio (HR) for replase with respect to negative expression of ARL4C. 4 Hazard ratio (HR) for death with respect to negative expression of ARL4C. 5 bold-italic value are statistically significant (p=0.05).
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resistant cells [32]. Additionally, to explore the function of DJ-1 in lung cancer, Clements et al examined its effect on global gene expression using Affymetrix GeneChip. After siRNA-mediated knockdown of DJ-1, ARL4C expression was significantly up-regulated, suggesting that ARL4C may be negatively regulated by DJ-1 [33]. DJ-1 was initially described as a Parkinson’s disease-related gene [34] and then a putative oncogene which could transform mouse NIH-3T3 cells in cooperation with Ras [35]. Moreover, DJ-1 was proved to be a key suppressor of PTEN [36]. Recent studies reported that DJ-1 was able to promote invasion and metastasis of pancreatic cancer cells by activating SRC/ERK/uPA [37].

To our knowledge, this is the first study suggesting that ARL4C may play an important role in ovarian cancer. Our understanding of ARL4C’s role in cancer is still quite limited. Much remains to be elucidated, including the relationship between DJ-1 and ARL4C in ovarian cancer and identification of downstream targets or pathways being involved in tumor cell migration and invasion. With a comprehensive understanding of ARL4C regulation and function, we may be able to explain how ARL4C is involved in ovarian cancer progression.

In summary, ARL4C was identified as a novel ovarian cancer-related gene by integrated molecular and genomic technologies. ARL4C was down-related in metastatic ovarian cancer cells. Its expression does not affect colony formation and cell proliferation, but negatively regulates cell motility. In vitro experiments suggest that ARL4C suppresses ovarian cancer cell migration, and clinical study indicates that high ARL4C expression is associated with better overall survival of ovarian cancer patients. Tumor analysis also suggests that ARL4C may serve as a candidate marker for ovarian cancer prognosis. More studies are needed to further elucidate the molecular mechanisms by which ARL4C effects in ovarian cancer progression.

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Disclosure of conflict of interest

We have no conflict of interest to declare.

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References


[8] Chen H, Wu X, Pan ZK and Huang S. Integrity of SOS1/EPS8/ABI1 tri-complex determines
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ovarian cancer metastasis. Cancer Res 2010; 70: 9979-9990.


