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Oncogenic Role of *eIF-5A2* in the Development of Ovarian Cancer

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ABSTRACT

Amplification of 3q26 is one of the most frequent chromosomal alterations in many solid tumors, including ovarian, lung, esophageal, prostate, breast, and nasopharyngeal cancers. A candidate oncogene to eukaryotic initiation factor 5A2 (*eIF-5A2*), a member of eukaryotic initiation factor 5A subfamily, has been isolated from a frequently amplified region at 3q26.2. In this work, the tumorigenic ability of *eIF-5A2* was demonstrated by anchorage-independent growth in soft agar and tumor formation in nude mice. Furthermore, antisense DNA against *eIF-5A2* could inhibit cell growth in ovarian cancer cell line UACC-1598 with amplification of *eIF-5A2* in form of double minutes. Cell growth rate in UACC-1598 was also inhibited when the expression level of *eIF-5A2* was decreased by the reduction of the copy number of double minutes. The correlation of *eIF-5A2* overexpression and clinical features of ovarian cancer was investigated using tissue microarray, and the result showed that *eIF-5A2* overexpression was significantly associated with the advanced stage of ovarian cancer. These findings suggest that *eIF-5A2* plays important roles in ovarian pathogenesis.

INTRODUCTION

Ovarian cancer is the leading cause of death from female gynecological malignancies in developed countries, and its incidence has been increasing in Asian countries such as China and Singapore recently (1). Because of its insidious onset, 70% of ovarian cancer patients were diagnosed at advanced stage, and the prognosis is very poor with a 5-year survival rate of <20% (2). Recurrent chromosomal changes in ovarian cancer have been well studied by comparative genomic hybridization and amplification of 3q26 is one of the most frequent alterations (3–6). Amplification of 3q26 has been also frequently detected in lung cancer (7), esophageal cancer (8), prostate cancer (9), breast cancer (10), and nasopharyngeal cancer (11). These studies suggest that 3q26 may contain one or more putative oncogenes, which play important roles in the development or the progression of various solid tumors, including ovarian cancer.

Recently, we have isolated a candidate oncogene eukaryotic initiation factor 5A2 (*eIF-5A2*) from 3q26.2 using chromosome microdissection and hybrid selection (12). Sequencing analysis showed that *eIF-5A2* shares a significant sequence homology (126 of 153, 82% amino acid identity) to eukaryotic initiation factor 5A (*eIF-5A*), including the domain needed for hypusine modification and the lysine 50 residue where the hypusine residue can be formed by posttranslational modification. Previous study showed that intracellular depletion of *eIF-5A* could cause the inhibition of cell growth (13). Other studies indicated that the inhibition of deoxyhypusine synthase, the enzyme involved in the hypusination reaction of *eIF-5A*, could inhibit Chinese hamster ovary cells proliferation (14), suppress the growth of HeLa cells, and *v-src*-transformed NIH3T3 cells (15), and induce apoptosis

(16). The proliferation-related function of *eIF-5A* supports that *eIF-5A2* is a candidate oncogene related to the development of ovarian cancer. Amplification and overexpression of *eIF-5A2* have been frequently detected in primary ovarian cancers and ovarian cancer cell lines (12, 17). Recently, Clement *et al.* (18) showed that *eIF-5A2* has an important role in eukaryotic cell survival. In this study, the tumorigenicity of *eIF-5A2*, the oncogenic role of *eIF-5A2* in signaling pathway, and the association between *eIF-5A2* overexpression and clinical features of ovarian cancer were investigated.

MATERIALS AND METHODS

Tumorigenic Ability of *eIF-5A2*. Mouse fibroblast cell line NIH3T3 was obtained from the American Type Culture Collection. Human liver cell line LO2 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, People's Republic of China). To evaluate the tumorigenic ability of *eIF-5A2*, *eIF-5A2* was cloned into expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) and transfected into NIH3T3 cell and LO2 cell independently using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Stable *eIF-5A2*-expressing clones were selected using Geneticin (Life Technologies, Inc.) at a concentration of 800 μ g/ml, and the expression level of *eIF-5A2* in each clone was determined by Northern blot analysis.

Soft agar assay was carried out by suspending 1×10^4 cells in 0.4% Seaplaque agar (BioWhittaker Molecular Applications, Rockland, ME) in DMEM supplemented with 10% FBS and seeded onto solidified 0.6% agar in a 6-well plate. The cells were replenished with fresh medium every 3 days, and colonies of at least four times as large as single cell were counted at day 21. Colony formation rate was calculated as colony number/seeded cell number $\times 100\%$. Triplicate independent experiment was done.

Tumor formation in nude mice was also performed with *eIF-5A2*-transfected NIH3T3 cells and LO2 cells. Each animal received single injection of 4×10^6 cells suspended in 0.2 ml of PBS. NIH3T3 or LO2 cells transfected with empty vector were injected on the left dorsal flank, and *eIF5A2*-transfected NIH3T3 or LO2 cells were injected on the right dorsal flank of the same animal. The animals were examined for tumor formation over a period of 1 month.

Reduction of Double Minutes (DMs) in UACC-1598. Ovarian cancer cell lines UACC-1598 were obtained from the Tissue Culture Core Service of the University of Arizona Comprehensive Cancer Center. UACC-1598 cells were grown in DMEM with 10% fetal bovine serum and exposed to 50 μ M hydroxyurea (HU) for 30 cell doublings and 100 μ M HU for 20 cell doublings, respectively. The parental UACC-1598 cell was used as a control. Genomic DNA was prepared, and the *eIF-5A2* copy number was determined by Southern blot analysis. The amount of total DNA was internally controlled by subsequent hybridization to a human β -actin probe. The ratio of *eIF5A2* to β -actin was then calculated using PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and compared with the control to yield relative percent. Expression of *eIF-5A2* was also analyzed by Northern blot hybridization. 28S and 18S rRNA in separating gel stained with ethidium bromide for the Northern blot were used as loading control.

To test cell growth rate, the HU-treated cells were washed, grown without HU for 48 h, and then seeded onto 24-well plate at a density of 1×10^4 cells/well and incubated for 1–7 days. The cell growth rate of HU-treated cells was compared with parental UACC-1598 cells using cell proliferation 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt kit (Roche, Mannheim, Germany) according to manufacturer's instructions.

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Antisense DNA against *eIF-5A2*. *eIF-5A2* was cloned into pcDNA3.1(+) vector in an antisense orientation. Approximately 2×10^6 UACC-1598 cells in a T25 flask were transfected with 12 μ g of pcDNA3.1(+) vector containing AS-*eIF-5A2* overnight using Lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions. After transfection, UACC-1598 cells were seeded onto 96-well plate at a density of 2×10^4 cells/well and incubated for 1–7 days. The cell proliferation rate of transfected cells was compared with the parental cell using cell proliferation 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt kit (Roche) according to manufacturer's instructions.

Southern, Northern, and Western Blot Analyses. For Southern blot analysis, 10 μ g of genomic DNA were digested with *EcoRI*, fractionated on 1% agarose gel, transferred to a nylon membrane, and hybridized with 32 P-labeled probes. For Northern blot analysis, 15 μ g of total cellular RNA were size fractionated on 1% agarose/2.2 M formaldehyde gel, transferred to a nylon membrane, and hybridized with 32 P-labeled probes. For Western blotting, 30 μ g of protein extract were resolved by 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride Hybond-P membrane (Amersham Pharmacia Biotechnology, Piscataway, NJ) by electroblotting. After blocking, the blot was probed with antibody, followed by treatment with secondary antibody. Immunoreactions were visualized by enhanced chemiluminescence according to the manufacturer's menu (Amersham Pharmacia Biotechnology).

Tissue Microarray (TMA) and Immunohistochemical Staining. For the construction of ovarian cancer TMA, the tumor cases encompassed 240 cases with histologically confirmed epithelial ovarian cancers from Cancer Institute, Sun Yat-Sen University (Guangzhou, People's Republic of China). The TMA

Table 1. Tumorigenicity test of *EIF-5A2*-expressing cells in nude mice

Cell lines	No. tumor formation/ no. tested nude mice	Tumor volume (mm ³) ^a
NIH3T3	0/10	
<i>eIF-5A2</i> -NIH3T3	0/10	
Vector-LO2	3/8	m1: 18; m2: 11; m5: 32
<i>eIF-5A2</i> -LO2	8/8	m1: 219; m2: 242; m3: 319; m4: 1015; m5: 167; m6: 319; m7: 118; m8: 292

^aTumor volume (V) was estimated from the length (l) and width (w) of the tumor using the formula: $V = (\pi/6) \times [(l + w)/2]^3$. Tumor volume of each nude mouse is listed.

was constructed as described previously (19). Briefly, a TMA instrument (Beecher Instruments, Silver Spring, MD) was used to create holes in a recipient paraffin block to obtain cylindrical core tissue biopsy samples with a diameter of 0.6 mm from all ovarian cancers and to transfer these biopsy samples to the recipient block at defined array positions. Two samples were selected from each case. Multiple sections (5- μ m thick) were cut from the TMA block and mounted on microscope slides.

Immunohistochemistry study was performed using the standard streptavidin-biotin-peroxidase complex method. Antigen retrieval was performed by treating the slide in 10 mM citrate buffer (pH 6.0) in a microwave for 5 min. A 1:2000 diluted polyclonal anti-*EIF-5A2* (SAGE BioVentures, San Diego, CA) antibody was used for *EIF-5A2* detection. Negative control was performed by replacing the primary antibody with blocking serum.

Statistical Analysis. Statistical analysis was performed with the SPSS software (SPSS Standard, version 8.0). The associations of *EIF-5A2* expression and clinical features of ovarian cancer in TMA were evaluated by Fisher's exact test. Significant correlation was considered when P was <0.05 .

RESULTS

Tumorigenic Ability of *eIF-5A2*. The tumorigenic ability of *eIF-5A2* was studied by anchorage-independent growth in soft agar and tumor formation in nude mice. *eIF-5A2* was cloned into expression vector pcDNA3.1(+) and then transfected into NIH3T3 and human liver cell line LO2. The expression level of *EIF-5A2* in transfected NIH3T3 and LO2 cells was determined by Northern blot hybridization (Fig. 1, A and B). Soft agar assay showed that *eIF-5A2* could obviously increase the colony formation in soft agar in both NIH3T3 and LO2 cells (Fig. 1, C and D). Tumor formation in nude mice was also performed with *eIF-5A2*-transfected NIH3T3 and LO2 cells. The result was summarized in Table 1. *EIF-5A2*-expressing NIH3T3 cells could not cause tumor formation in nude mice. However, *EIF-5A2*-expressing LO2 cells exhibited strong tumor formation ability in nude mice. In all eight nude mice that received injections of *EIF-5A2*-expressing LO2 cells, the tumor appeared in 10–14 days, and the tumor volume ranged from 118 to 1015 mm³ after 1 month (Fig. 1, E and F, and Table 1). Tumor formation was also observed in 3 of 8 empty vector-transfected LO2 cells with tumor volume 11–32 mm³. To confirm whether the tumor formation in nude mice was caused by *eIF-5A2* transfection, expression of *EIF-5A2* in both tumors induced by *EIF-5A2*-expressing LO2 and vector-transfected LO2 cells was examined by anti-*EIF-5A2* antibody. Cytoplasmic expression of *EIF-5A2* was detected in all tumors induced by *EIF-5A2*-expressing LO2 cells but not in tumors induced by vector-transfected LO2 cells (data not shown).

Fine Mapping DMs in UACC-1598. High copy number amplification of *EIF-5A2* has been detected in an ovarian cancer cell line UACC-1598 in the form of DMs (12). To identify all possible amplified genes within the amplicon, a bacterial artificial chromosome contig covering the 3q26.2 region was established by NCBI BLAST search. Boundaries of the amplicon were determined by Southern blot hybridization using DNA sequences from bacterial artificial chromosome clone as probes (Fig. 2). The distance between boundaries (P2 and P3) of the amplicon is ~ 388 kb, and *eIF-5A2* was the only known

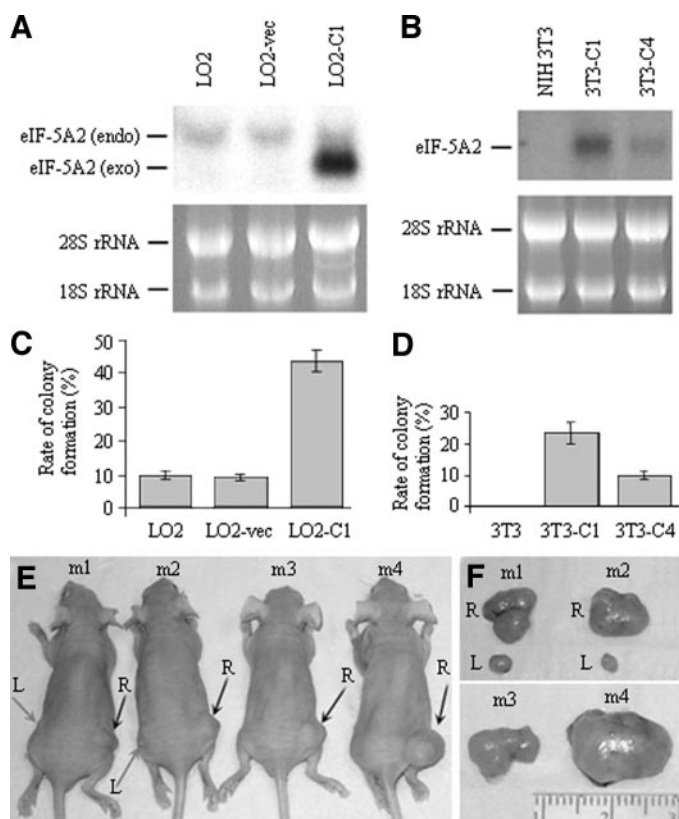
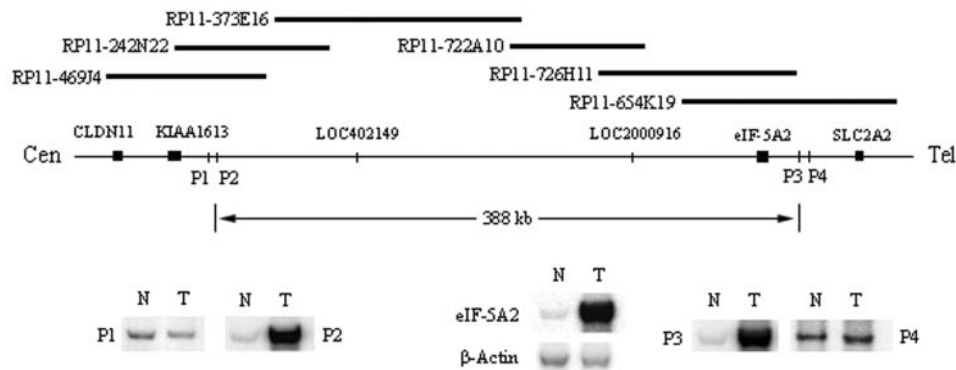


Fig. 1. Oncogenic property of eukaryotic initiation factor 5A2 (*eIF-5A2*). A, expression of endogenous and exogenous *eIF-5A2* in LO2, vector-transfected LO2 (LO2-vec), and *eIF-5A2*-transfected LO2 (LO2-C1) cells detected by Northern blot hybridization. 28S and 18S rRNA were used as loading control. B, expression of *eIF-5A2* in NIH3T3, *eIF-5A2*-transfected NIH3T3 clone 1 (3T3-C1), and clone 4 (3T3-C4) detected by Northern blot hybridization. C, rate of colony formation in soft agar detected in LO2, vector-transfected LO2, and *eIF-5A2*-transfected LO2 cells. D, rate of colony formation in soft agar observed in NIH3T3, 3T3-C1, and 3T3-C4 cells. E, examples of tumors formed in nude mice (m1–m4) at the time of 1 month after injection of *EIF-5A2*-expressing LO2 cells (right dorsal flank, indicated by dark arrows) and vector-transfected LO2 cells (left dorsal flank, indicated by light arrows). F, the same tumors from these animals in Fig. 2A were dissected. Tumors at top and bottom rows were derived from *EIF-5A2*-expressing LO2 and control vector-transfected LO2 cells, respectively.

Fig. 2. Fine map of double minutes in UACC-1598. Horizontal bars represent bacterial artificial chromosome clones covering the amplicon of double minutes at 3q26.2. Boundaries of the amplicon (P2 and P3) were determined by Southern blot hybridization with probes P1–P4, which are shown at the bottom (*n* = normal DNA, T = tumor DNA from UACC-1598). Genes within or close to the amplicon are indicated above the fine mapping line.



gene within the amplicon based on BLAST search. Two predicted genes (LOC402149 and LOC2000916) were found within the amplicon (Fig. 2). To evaluate the expression level of these two predicted genes, reverse transcription-PCR was used to amplify these two genes. However, no PCR product was obtained.

Inhibition of Cell Growth by Reducing DMs. To determine whether the amplification of *eIF5A2* could accelerate cell proliferation like *eIF-5A*, the correlation of reduction of *eIF-5A2* copy number and cell growth rate was studied. Low concentration of HU was added into the culture medium to reduce the DMs in UACC-1598 because HU can eliminate extrachromosomal DMs in culture cells (20). After 20 passages of exposure to 100 μM and 30 passages to 50 μM HU, the copy number of *eIF5A2* was reduced to 78 and 45%, respectively, compared with those in the parental UACC-1598 cells (Fig. 3A). The expression of EIF-5A2 was also reduced to 66 and 30%, respectively, compared with those in the parental cells (Fig. 3A). Cell growth assay showed that reduction of DMs containing *eIF-5A2* in UACC-1598 led to a decreased cell growth rate *in vitro* (Fig. 3B).

Inhibition of Tumor Cell Growth by Antisense DNA against *eIF-5A2*. Full-length antisense DNA sequence against *eIF-5A2* was cloned into expression vector pcDNA3.1(+) and then transiently

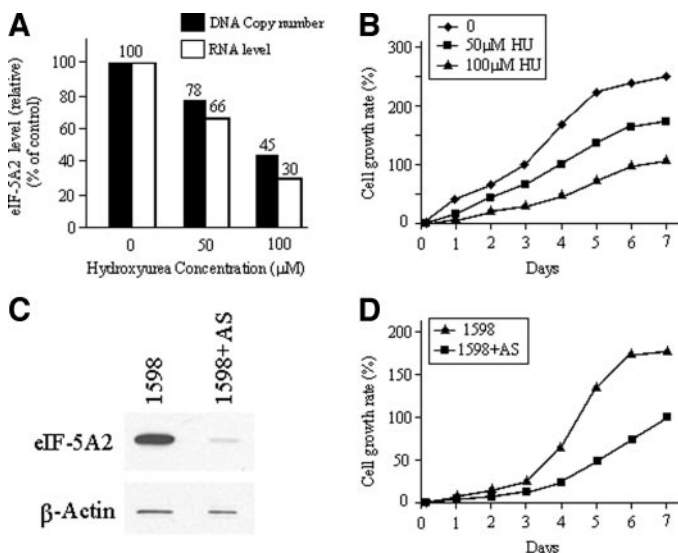


Fig. 3. A, the copy number of eukaryotic initiation factor 5A2 (*eIF5A2*) and RNA expression of EIF-5A2 were reduced as the reduction of double minutes in UACC-1598 by hydroxyurea (HU) treatment. B, reduction of double minutes containing *eIF-5A2* in UACC-1598 leads to a decreased cell growth rate in culture cells. C, expression of EIF-5A2 in UACC-1598 cells transfected with AS-*eIF-5A2* (1598+AS) and the parental UACC-1598 cells (1598) detected by Western blot analysis. β -Actin is used as loading control. D, the cell growth rate of AS-*eIF-5A2*-transfected 1598-AS cell was obviously decreased compared with the parental 1598 cell.

transfected into UACC-1598 cells. Western blot analysis showed that the expression of EIF-5A2 was effectively blocked by AS-*eIF-5A2* (Fig. 3C). Relative to β -actin, expression of EIF-5A2 in UACC-1598 cells transfected with AS-*eIF-5A2* was decreased 80% compared with that in the parental UACC-1598 cells. As a result, the cell growth rate of UACC-1598 cells transfected with AS-*eIF-5A2* was obviously decreased compared with the parental UACC-1598 cells (Fig. 3D).

Correlation of EIF-5A2 Overexpression and Clinical Features of Ovarian Cancer. The correlation of EIF-5A2 overexpression and clinical features of ovarian cancer was studied by TMA with 240 epithelial ovarian cancers. Using immunohistochemical staining with anti-EIF-5A2 antibody, informative staining was observed in 211 of 240 (88%) cases. The noninformative samples included lost samples, unrepresentative samples, and samples with too few tumor cells. On the basis of the staining density, cytoplasmic expression of EIF-5A2 could be divided into negative, weak, moderate, and strong. Because moderate and strong expression of EIF-5A2 could not be observed in 7 normal ovary (not included in TMA), negative/weak and moderate/strong staining were defined as normal expression and overexpression, respectively. Normal expression and overexpression of EIF-5A2 were detected in 89 (42.2%) cases and 122 cases (57.8%), respectively. The association between EIF-5A2 expression and clinical features of ovarian cancer, including patient's age, tumor's histological type, and tumor's Silverberg grade, was analyzed. A significant association between EIF-5A2 overexpression and tumor's International Federation of Gynecology and Obstetrics stage was observed. The frequency of overexpression of EIF-5A2 in advanced International Federation of Gynecology and Obstetrics stages (III and IV; 85 of 123, 69.1%) was significantly higher than that in earlier stages (I and II) (37 of 88, 42.0%; $P < 0.001$, Fisher's exact test). Fig. 4 shows the examples of normal expression of EIF-5A2 in normal ovary and overexpression of EIF-5A2 in ovarian cancer. No significant association was found between EIF-5A2 overexpression and other clinical features.

DISCUSSION

Amplification of 3q26 is one of the most frequent chromosomal alterations in ovarian cancer, lung cancer, esophageal cancer, prostate cancer, breast cancer, and nasopharyngeal cancer, suggesting an oncogene(s) localized in this region playing important roles in carcinogenesis. Recently, one candidate oncogene *eIF-5A2*, which shares 82% amino acid sequence with *eIF-5A*, has been isolated from 3q26.2. A number of studies have shown the involvement of EIF-5A in cell growth, cell proliferation, and against apoptosis (13–16). As the result of its similarity to EIF-5A, it is highly possible that EIF-5A2 may also play important roles in carcinogenesis.

In the present study, the oncogenic role of *eIF-5A2* was supported

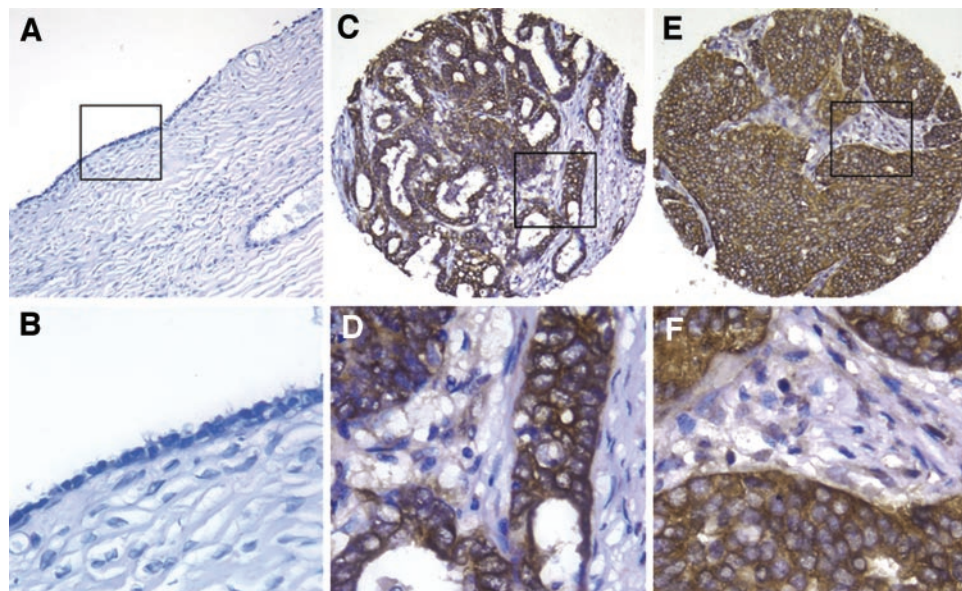


Fig. 4. Examples of immunohistochemical staining of EIF-5A2 in ovarian cancer tissue microarray with anti-EIF-5A2 antibody. Negative staining was observed in epithelial cells of normal ovary ($\times 100$; A) and higher magnification ($\times 400$) from the area of the box in A (B). C and E, cytoplasmic overexpression of EIF-5A2 was detected in two ovarian cancer cases ($\times 100$). D and F, higher magnification ($\times 400$) from the area of the box in C and E, respectively.

by the following evidence: (a) cell growth in ovarian cancer cell line UACC-1598 was inhibited when *eIF-5A2* expression level was decreased by reducing the copy number of DMs containing *eIF-5A2*; (b) treatment of UACC-1598 cells with AS-EIF-5A2 obviously decreased the cell growth; (c) anchorage-independent growth in soft agar was observed in *eIF-5A2*-transfected NIH3T3 and LO2 cells; (d) tumor formation in nude mice was induced in *eIF-5A2*-transfected LO2 cells; and (e) overexpression of EIF-5A2 was significantly associated with the advanced stages of ovarian cancer.

The size of amplicon of the DMs is ~ 388 kb and *eIF-5A2* is the only known gene within the amplicon. It is possible that some other genes within the amplicon are amplified and related to the pathogenesis of ovarian cancer. On the basis of the BLAST search, at least two predicted genes have been mapped within the amplicon. Reverse transcription-PCR results showed that both genes could be pseudogenes.

Other than *eIF-5A2*, *PIK3CA* has been implicated as a candidate oncogene at 3q26 (21). Overexpression of *PIK3CA* has been also associated with the pathogenesis of cervical cancer and head and neck cancer (22, 23). It is possible that EIF-5A2 may cooperate with *PIK3CA* to promote ovarian carcinogenesis. Better understanding of the physiological and pathophysiological functions of EIF-5A2 may lead to a much more effective management of ovarian cancer with the amplification of the *eIF-5A2*, via early detection, precise prognostication, and molecularly targeted treatment.

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