

# The prognostic significance of elongation factor eEF1A2 in ovarian cancer <sup>☆</sup>

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Received 9 July 2007

Available online 31 December 2007

## Abstract

**Objective.** To determine whether eukaryotic elongation factor 1 alpha 2 (eEF1A2), a transforming gene previously shown to be highly expressed in primary human ovarian tumours, is a prognostic marker.

**Methods.** We have used an antibody specific for eEF1A2 to measure eEF1A2 protein expression in 500 primary ovarian tumours in a tissue microarray. We have also ectopically expressed eEF1A2 in SK-OV-3 cells, a clear cell carcinoma line that does not normally express eEF1A2.

**Results.** We have shown that eEF1A2 has high expression levels in ~30% of all primary ovarian tumours. 50% of serous tumours, 30% of endometrioid, 19% of mucinous and 8% of clear cell tumours highly express eEF1A2. Ectopic expression of eEF1A2 in the SK-OV-3 clear cell carcinoma line enhances their *in vitro* proliferative capacity and ability to form tumour-like spheroids in hanging drop culture. Expression of eEF1A2 did not alter sensitivity to anoikis, cisplatin, or taxol. In serous cancer, eEF1A2 is an independent prognostic marker for survival and high eEF1A2 protein expression was associated with increased probability of 20-year survival.

**Conclusions.** eEF1A2 is highly expressed in ovarian carcinomas. Its expression enhances cell growth *in vitro*, and eEF1A2 expression is likely to be a useful ovarian cancer prognostic factor in ovarian cancer patients with serous tumours.

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**Keywords:** eEF 1A2; Protein translation; Oncogene; Spheroid; TMA; Prognostic factor

## Introduction

The identification of genetic abnormalities that occur during ovarian cancer development is a necessary part of understanding its root causes. We have previously identified eEF1A2 (eukaryotic elongation factor 1 alpha 2) as a putative ovarian cancer oncogene by virtue of its high expression and gene amplification in primary human ovarian tumours [1,2]. In addition, the eEF1A2 gene is transforming: ectopic eEF1A2 expression in rodent fibroblast cells allows these cells to grow in an anchorage independent fashion and enhances their tumourigenicity when xenografted in nude mice [2].

eEF1A2 is one of two isoforms of the eukaryotic elongation factor 1 alpha: eEF1A1 and eEF1A2 [3,4]. eEF1A proteins bind amino-acylated tRNAs and recruit them to the ribosome during

the elongation phase of protein translation [1,5]. In addition to its role in protein elongation, eEF1A2 regulates a multitude of other cellular processes [1,2,6,7]. For example, eEF1A2 is reported to be an inhibitor of caspase 3-dependent apoptosis [8] and deletion of the mouse eEF1A2 homologue, *Eef1a2*, results in immunodeficiency, elevated lymphoid apoptosis and death by 30 days of age [9–11]. eEF1A2 also regulates cell signaling and we have previously reported that eEF1A2 binds and activates the PI4KIII $\beta$  lipid kinase [7]. eEF1A2 also stimulates the Akt serine/threonine protein kinase and activates Akt-dependent cell migration and actin remodeling [6].

The two eEF1A isoforms have markedly different tissue-specific expression patterns: eEF1A1 is expressed ubiquitously while eEF1A2 expression is restricted to the brain, heart and skeletal muscle [1,3,4]. Moreover, eEF1A2 is highly expressed in a subset of ovarian, lung and breast tumours, suggesting an important role for eEF1A2 in oncogenesis [2,12–14]. The role of eEF1A2 in cancer outcome is likely to be complex; high eEF1A2 expression is a marker for good outcome in breast cancer [13] but a marker for poor survival in lung cancer [12]. It is not yet known

<sup>☆</sup> This work was supported by the National Cancer Institute of Canada, with funds from the Canadian Cancer Society.

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whether eEF1A2 expression has prognostic significance in ovarian cancer.

In this report, we show that ~30% of primary human ovarian tumours have high eEF1A2 protein expression. eEF1A2 is highly expressed in 50% of serous and 30% of endometrioid tumours but in a much smaller fraction of clear cell and mucinous carcinomas. Expression of eEF1A2 in ovarian clear cell carcinoma cells enhances *in vitro* proliferation and their ability to form tumour-like spheroid cultures. eEF1A2 expression is also a significant predictor of 20-year survival in ovarian cancer of the serous type.

## Materials and methods

### Ovarian tumour tissue microarray (TMA)

Tissue arrays were constructed from 500 archival formalin-fixed, paraffin-embedded ovarian tumour samples from Vancouver General Hospital. These included a variety of high and low grade tumours (see Results for detailed information). The tissue microarrays (TMA) were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD) as described previously [15]. Briefly, the recipient block had holes created by the instrument with defined array coordinates and a stylet was used to transfer the tissue cores to the recipient block.

### Immunohistochemistry

The TMA sections underwent immunostaining on a Ventana Discovery Instrument (Ventana Medical systems, Tucson, Arizona) using a DAB Map Kit (HRP labeled Biotin-Streptavidin System). The staining was done as outlined in [13]. Briefly, the staining steps were: deparaffinization, heat induced antigen retrieval with EDTA pH 8.0 (24 min), hydrogen peroxide quenching 3% H<sub>2</sub>O<sub>2</sub> (8 min), eEF1A2 antibody (1:100 dilution) (32 min), biotinylated universal secondary antibody (32 min), streptavidin–biotin peroxidase complex (16 min), DAB (8 min), counterstain with hematoxylin (4 min).

### Evaluation of eEF1A2 protein expression

The staining levels of eEF1A2 were evaluated as previously described [16,17]. Briefly, the images of the tissue cores were scanned using a Bacus Laboratories Inc. Slide Scanner (BLISS) (Bacus Laboratories, Inc., Lombard, IL). WebSlide Browser v.3.98 (Bacus Laboratories, Inc., Lombard, IL) was used to view the images of the arrays and to assess the individual core images. H&E slides were scanned along with immunohistochemical ones and used as a reference to determine the expression of protein to the specific breast carcinoma structures. Images are available through <http://www.gpecimage.ubc.ca/tma/webviewer.php>. Scoring of the eEF1A2 immunostaining was performed semi-quantitatively, using digital images and 22-in. monitor with hardware color calibration capabilities. Staining was considered to be negative (0) if no staining was seen in the tumour, weak positive (1+), moderate (2+) and strong (3+), depending on the intensity of the staining in the cytoplasm. Scores were entered into a standard Excel worksheet (Microsoft Excel, Microsoft, Redmond, WA) with a sector map matching each TMA section. Scores for duplicate cores were consolidated to a single value per case using an Excel macro developed by DT. If there were discrepant scores for the two, the high value was accepted for the case. Cases were not included if there was no tumour tissues in the core or if the core was cut through. Original scoring tables were deconvoluted together with the core identification file using Deconvolutor 1.10 [18], and the resulting table files imported into SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

### Cell culture and western blotting

SK-OV-3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown according to their instructions. Cells were transfected with 5 µg *EEF1A2* (with C-terminal V5 tag) plasmid and 15 µl Super-Fect (Qiagen) per 60 mm dish and 0.4 mg/ml Zeocin (Invitrogen) was

used for selection. Cells were then lysed using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl; pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA); pH 7.0, 150 mM NaCl) supplemented with 1% aprotinin, 1 mg/ml leupeptin, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mg/ml pepstatin in ethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) in dimethylsulfoxide (DMSO). Protein was quantified using Bradford protein assay (Pierce, Rockford, IL, USA) as per the manufacturer's instructions. Approximately 20 µg of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. Anti-V5-horseradish peroxidase (HRP) (Invitrogen) and actin (Sigma) antibodies were used according to manufacturers' instructions.

### Cell proliferation assay and multi-cellular spheroid culture

Cell proliferation assays were performed by culturing cells as mentioned previously. At indicated time points cells were trypsinized and counted by trypan blue exclusion. This was performed in triplicate. The multi-cellular spheroids were cultured as outlined by Kelm et al. [19]. Briefly, 15 µl droplets containing 1000 SK-OV-3 cells were placed on the lids of non-adherent, bacterial grade polystyrene Petri dishes (Starstead). Lids were then inverted over Petri dishes filled with 10 ml 1× phosphate buffered saline (PBS).

### Anoikis and cell death

To induce anoikis, cells were shaken at 40 rpm in standard tissue culture dishes on an S-500 orbital shaker (VWR) in a 37 °C, 5% CO<sub>2</sub> incubator. Cells were counted using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA) at set time points. Sensitivity of cells to cisplatin and paclitaxel were performed by adding serial dilutions of either cisplatin or taxol to cells and determining cell number using trypan blue exclusion counting. The IC<sub>50</sub> was calculated as the drug dose that reduced surviving cell abundance by 50%.

### Flow cytometry

In order to analyze the levels of apoptosis by flow cytometry, 500 000 SK-OV-3 cells were shaken at 40 rpm in standard tissue culture dishes on an S-500 orbital shaker (VWR) in a 37 °C, 5% CO<sub>2</sub> incubator. Cells were then removed from the culture dishes by pipetting. Control SK-OV-3 cells were trypsinized from standard tissue culture dishes. Cells were washed with 1× PBS then resuspended in 500 µl 1× Binding Buffer (Sigma). Cells were then stained with 2.5 µl of a 50 µg/ml Annexin V FITC conjugate (Sigma) and analyzed by flow cytometer, FC500 Flow Cytometer Beckman Coulter (Beckman Coulter, Fullerton, CA).

### Statistical analysis

Univariable survival analysis was performed using the Kaplan–Meier method and log-rank test. Multivariable survival analysis was performed using the proportional-hazards model. The level of significance for all statistical tests was defined as  $p < 0.05$ . All survival analyses were performed using JMP v6.0.3 (SAS Institute, Cary, NC, USA).

## Results

### eEF1A2 expression in ovarian cancer

We have previously reported that eEF1A2 mRNA is not detectable in normal ovarian epithelium but is expressed in a fraction of human ovarian tumours [1,2]. To further characterize the expression of eEF1A2 in ovarian cancer, we analyzed eEF1A2 protein expression in 500 ovarian tumours on a tumour microarray. Of the tumours sampled, 42% of those were in stage I, 41% were stage II and 17% were stage III and the entire patient cohort had no macroscopic residual disease after the surgery. The distribution of the subtypes of the tumour samples are as follows: 42% Serous, 26% Clear cell, 25% Endometrioid and 7% Mucinous. The age of

the patients assayed range from 9258 to 32,502 days with a mean age of  $21,197 \pm 4665$  days. The creation and validation of the eEF1A2 antibody used is described elsewhere [13]. Staining was categorized as negative, weak, moderate or high. Representative photographs of the 4 categories are shown in Fig. 1. Overall, 32% of the tumours (159/500) tested have high expression of eEF1A2. As shown in Table 1, the tumour types with the most frequent expression of eEF1A2 were serous and endometriod tumours, with 50% and 30% of these tumours having high levels of eEF1A2 expression respectively. 19% of the mucinous tumours and 8% of clear cell tumours had high expression of eEF1A2.

*eEF1A2 increases cell proliferation in vitro*

In order to determine the role that eEF1A2 might have in ovarian tumour development, we ectopically expressed eEF1A2 in SK-OV-3 cells. SK-OV-3 cells are derived from a human clear cell ovarian adenocarcinoma and do not endogenously express eEF1A2 (Fig. 2A). eEF1A2 protein expression in three independent SK-OV-3 clones is shown in Fig. 2A. As shown in Fig 2B, SK-OV-3 variants that express eEF1A2 proliferate at a faster rate than their wild type and vector counterparts (Fig. 2B), eEF1A2-negative parental and empty vector controls had a doubling time of between 53 and 61 h. On the other hand, the doubling time for

Table 1

Evaluation of eEF1A2 expression in ovarian cancer

| Histological type   | eEF1A2 expression   | Percentage of tumours (%) |
|---------------------|---------------------|---------------------------|
| Serous (n=212)      | High                | 50                        |
|                     | None, low, moderate | 50                        |
| Endometriod (n=125) | High                | 30                        |
|                     | None, low, moderate | 70                        |
| Mucinous (n=31)     | High                | 19                        |
|                     | None, low, moderate | 81                        |
| Clear cell (n=132)  | High                | 8                         |
|                     | None, low, moderate | 92                        |

eEF1A2-expressors was substantially less, between 22 and 39 h depending on the cell line. This indicates that expression of eEF1A2 increases the cells *in vitro* proliferative capacity.

*Expression of eEF1A2 increases the rate of spheroid formation*

We next wanted to determine whether eEF1A2 can affect other aspects of *in vitro* cell growth. Multi-cellular spheroids (MCS) have been used as an *in vitro* model system of avascular tumour development [19–24]. When SK-OV-3 cells were grown as MCS by the hanging drop method, they form a three-dimensional sphere approximately 2–3 days after culture initiation. As shown in Fig 3A, SK-OV-3 cells that express eEF1A2 form MCS more rapidly than vector counter parts (Fig. 3A). To quantitate this difference, we estimated the size of each spheroid by counting the

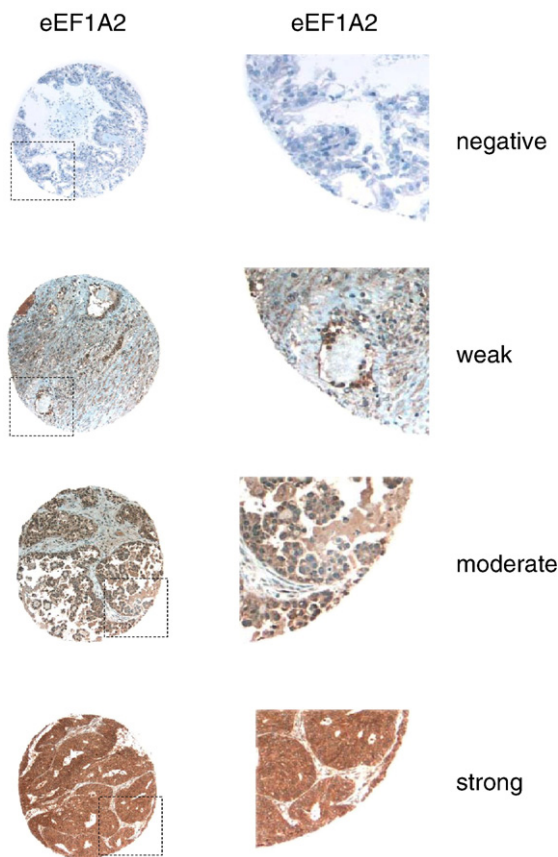


Fig. 1. eEF1A2 expression in primary ovarian tumours in a tissue microarray. Representative examples of eEF1A2 immunostaining in tumours classified as showing negative, weak, moderate and strong expression of eEF1A2. The right column is a higher magnification view of the boxed square of the left column.

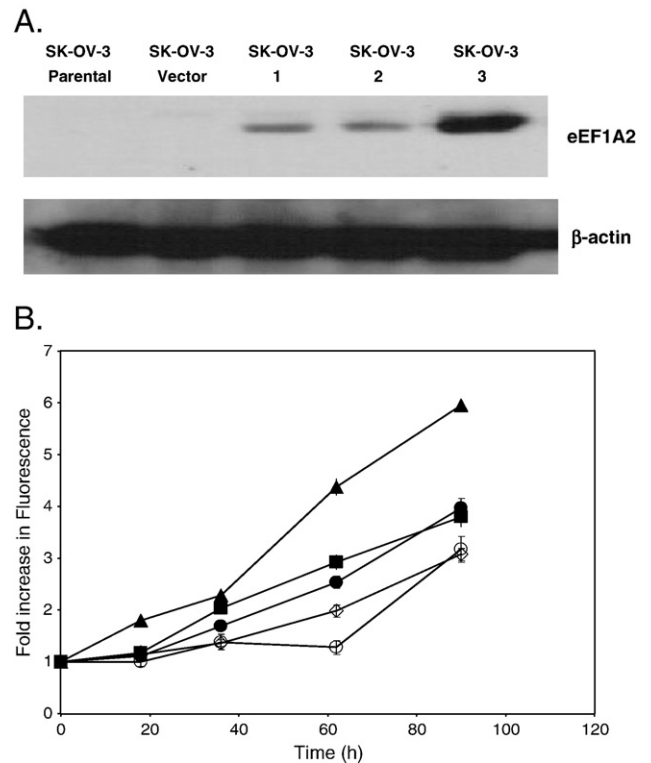


Fig. 2. Expression of eEF1A2 enhances cell proliferation. (A) Western blot of eEF1A2 expression. Parental and Vector controls do not express eEF1A2 while SK-OV-3 cell lines E1, E2 and E3 show detectable eEF1A2 protein expression. (B) eEF1A2-expressing cells (closed symbols) proliferate at a faster rate than the Parental and Vector controls (open symbols).

number of pixels in photographs of each spheroid. As shown in Fig. 3B, the vector controls formed spheroids which were approximately 104,000 pixels in size (Fig. 3B). On the other hand, eEF1A2-expressors have significantly ( $p < 0.001$ , ANOVA) smaller spheroids, 65,000 to 74,000 pixels in size. This indicates that the expression of eEF1A2 in these cell lines is enhancing the adhesive interactions responsible for spheroid formation [23,24].

*Expression of eEF1A2 does not affect resistance to anoikis or chemotherapeutics*

As eEF1A2 has previously been linked to an increased resistance to apoptosis [8,11], we next determined whether or not expression of eEF1A2 altered cellular susceptibility to anoikis. Anoikis is a specific type of apoptosis that occurs when cells lose cell–matrix adhesion [25–27]. We used shaking culture to induce

anoikis and as shown in Fig. 4A, SK-OV-3 cells that express eEF1A2 do not show a substantial difference in overall survival in shaking culture relative to controls. We also measured apoptosis in these cells using Annexin V and find no substantial difference in apoptosis between eEF1A2 expressing and controls SK-OV-3 cells (Fig. 4B). We also investigated whether eEF1A2 might alter the cytotoxicity of cisplatin or taxol. As with anoikis, eEF1A2 expression does not detectably affect the cytotoxicity of either cisplatin or taxol (Table 2).

*Expression of eEF1A2 indicates good long-term prognosis in serous tumour types*

We next investigated whether eEF1A2 expression had prognostic significance for long-term survival in ovarian cancer. Based on the assumption that eEF1A2 is an intensity-based marker, we

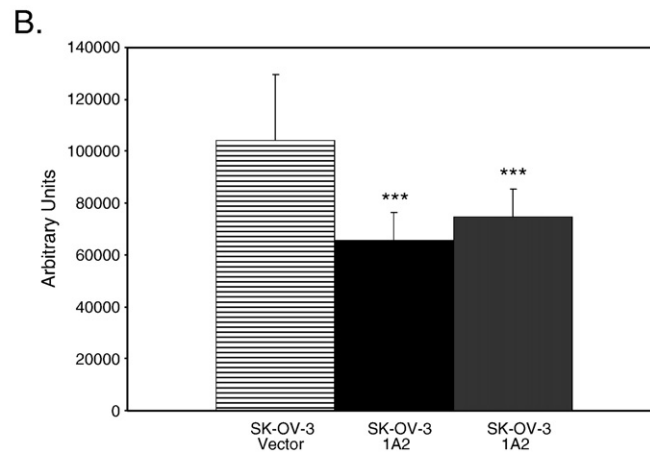
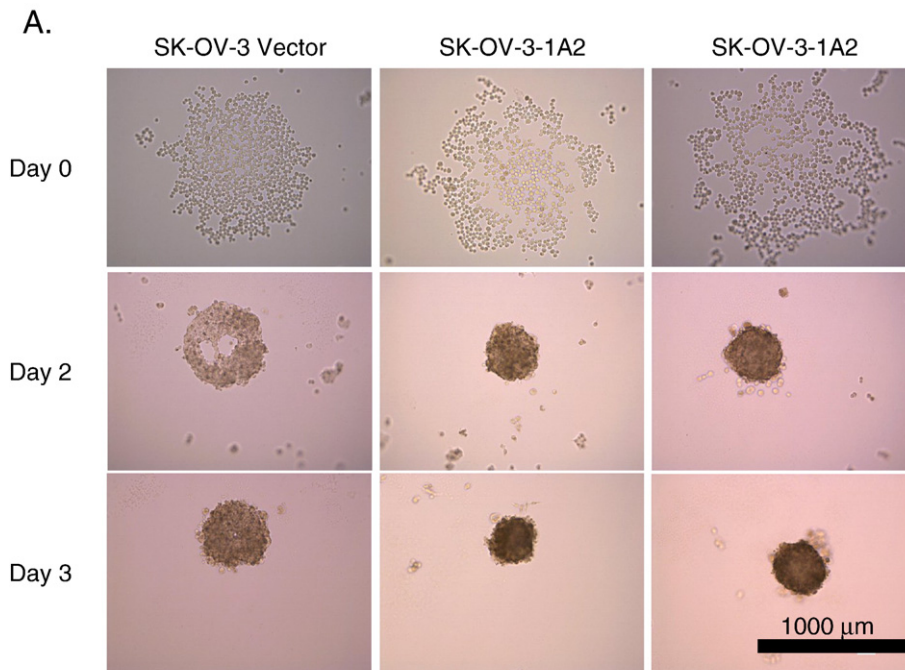


Fig. 3. Expression of eEF1A2 enhances spheroid formation. (A) Representative photographs of SK-OV-3 cells forming tumour-like spheroids in hanging drop culture. Cells (E1 and E3) that express eEF1A2 form spheroids more rapidly than their vector counterparts. (B) Aggregation was quantified after 2 days to quantitate the approximate cross sectional area of the spheroids. Data is the mean and standard deviation of three independent experiments with triplicates measurements. E1 and E3 spheroids are significantly smaller than the vector controls. Significance is indicated by \*\*\* ( $p < 0.001$ , ANOVA).

used recursive partitioning, an unsupervised procedure, to divide the tumour cohort into two groups to maximize observable differences in mean disease specific survival time. We portioned the tumours cohort in the high expressors (3) and grouped the negative to moderate staining (0–2) tumours together. When not separated by tumour type, patients whose ovarian tumours had

high expression of eEF1A2 had approximately the same 20-year survival outcome as their eEF1A2 low or negative group (Fig. 5A), 50% survival in both groups is approximately 12 years. However, in women with serous tumours, high expression of eEF1A2 was associated with significantly ( $p < 0.01$ , log rank test) increased 20-year survival probability (Fig. 5B). For example, at 10 years after

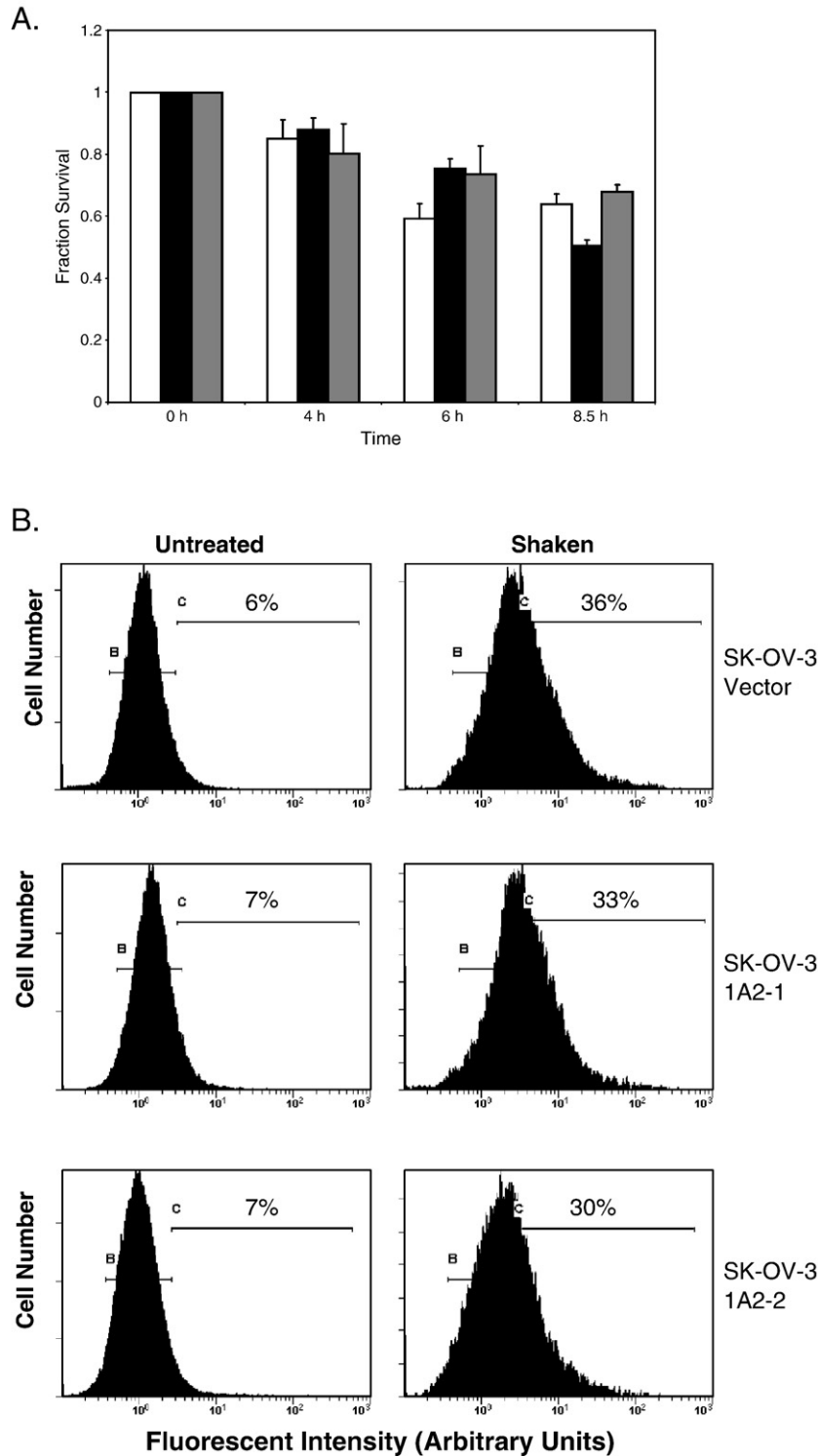


Fig. 4. Expression of eEF1A2 does not detectably affect anoikis. (A) SK-OV-3 Vector only cells (white bar) and E1 (black) and E3 cells (gray) were induced to undergo anoikis and cell number counted at set time points. Data is the mean and standard deviation of triplicate cell counts. (B) Annexin V staining was measured in SK-OV-3 vector and eEF1A2-expressors after 20 h in shaking culture. Figure is representative of three independent experiments.

Table 2  
Effect of eEF1A2 on cisplatin and taxol sensitivity

| Cell line        | Cisplatin IC <sub>50</sub> (mM) | Taxol IC <sub>50</sub> (mM) |
|------------------|---------------------------------|-----------------------------|
| SK-OV-3-Parental | 82.5                            | 35.3                        |
| SK-OV-3-Vector   | 78.8                            | 38.6                        |
| SK-OV-3-1A2      | 75.2                            | 42.8                        |
| SK-OV-3-1A2      | 83.4                            | 35.6                        |
| SK-OV-3-1A2      | 80.5                            | 39.6                        |

diagnosis, the surviving fraction of women with eEF1A2<sup>high</sup> tumours was ~50%, compared to ~30% in the others. Proportional hazards analysis shows that eEF1A2 is an independent prognostic marker in the serous subtype when age, stage and Silverberg grade are included in the model (Table 3). Other groupings do not lead to statistically significant differences in survival probability (not shown). eEF1A2 was not a significant prognostic marker for endometriod, clear cell or mucinous cancers (not shown).

## Discussion

In this report we show that *EEF1A2*, a gene not expressed in normal ovary, is highly expressed in ~30% of human ovarian

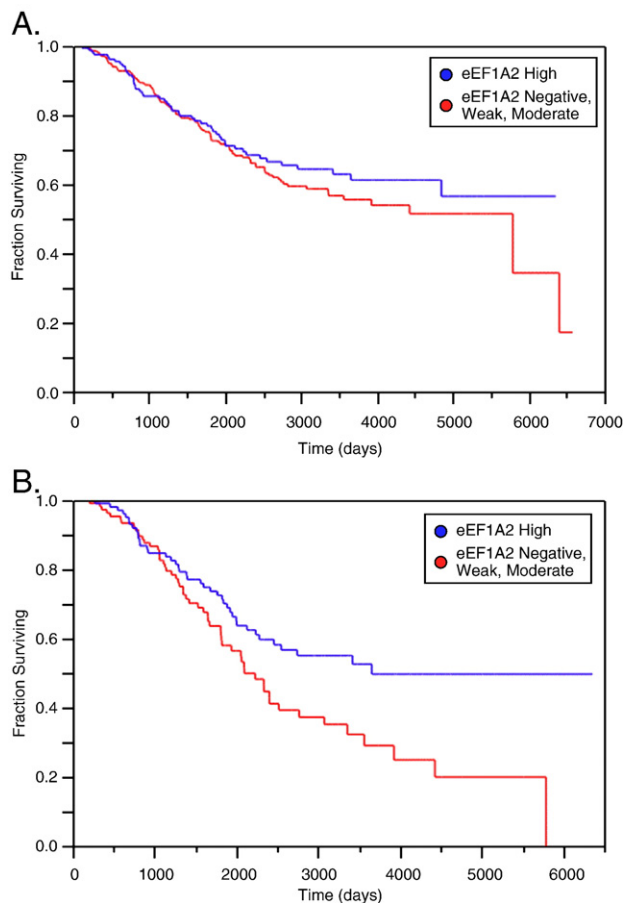


Fig. 5. Prognostic significance of eEF1A2. Fractional survival comparison of patients whose ovarian tumours showed negative, weak and moderate expression of eEF1A2 (red line) and with high expression (blue line) as a function of time. (A) Fractional survival is similar between the two populations when all ovarian tumour types are examined. (B) Long-term fractional survival is increased in serous tumours when they highly express eEF1A2.

Table 3  
Multivariable analysis of eEF1A2

| Term             | Levels | Risk ratio       | P value |
|------------------|--------|------------------|---------|
| eEF1A2           | 0      | 1.0 [1.0–1.0]    | 0.0210  |
|                  | 1      | 0.62 [0.41–0.93] |         |
| Stage            | I      | 0.81 [0.57–1.13] | 0.0002  |
|                  | II     | 0.68 [0.50–0.92] |         |
|                  | III    | 1.0 [1.0–1.0]    |         |
| Silverberg grade | 1      | 0.92 [0.45–1.59] | 0.6880  |
|                  | 2      | 1.15 [0.78–1.78] |         |
|                  | 3      | 1.0 [1.0–1.0]    |         |
| Age              | –      | NR               | 0.2414  |

tumours. 50% and 30% of the serous and endometriod tumours have high expression of eEF1A2, while only 19% and 8% of mucinous and clear cell tumours, respectively, express eEF1A2. This high expression of eEF1A2 in ovarian tumours implies that eEF1A2 expression has some causal role in neoplastic development. We have previously reported that eEF1A2 transforms rodent fibroblasts and enhances their tumourigenicity in nude mice [2]. Consistent with these observations, we find that eEF1A2 expression in SK-OV-3 clear cell carcinoma cells increases their *in vitro* growth rate. eEF1A2 also enhances the ability of SK-OV-3 cells to form *in vitro* spheroids in hanging drop culture. The hanging drop spheroid culture provides a model for studying avascular tumour regions *in vitro* [22]. eEF1A2 expression causes cells to aggregate into spheroids more quickly than their vector control, suggesting that eEF1A2 may be contributing to primary tumour formation *in vivo*.

Given the ability of eEF1A2 to enhance cell growth *in vitro*, it is therefore somewhat of a paradox that eEF1A2 expression predicts good survival probability in serous cancers. We have previously reported that eEF1A2 expression similarly marks good survival probability in breast cancer [13]. There are several possible explanations for the ability of eEF1A2 to mark good survival. eEF1A2 could affect chemotherapy resistance or alter primary tumour dissemination, both of which have important roles in controlling ultimate clinical outcome in ovarian cancer [28,29]. However, we do not find that eEF1A2 expression has a detectable effect on anoikis or sensitivity to cisplatin or taxol. This was a surprise to us since it has been previously reported that ectopic expression of S1, the rat eEF1A2 homologue, inhibits caspase 3-dependent apoptosis [8,11]. In addition, homozygous deletion of the mouse eEF1A2 gene, *Eef1a2*, increases lymphoid apoptosis [10]. While these reports indicate that eEF1A2 inhibits apoptosis, we find no effect on apoptosis in SK-OV-3 cells. With respect to tumour dissemination, we have previously reported that eEF1A2 can enhance *in vitro* cell migration and invasion of breast cancer cells [6]. At first glance it would therefore seem probable that eEF1A2 should enhance *in vivo* dissemination of the primary tumour in the peritoneal cavity. However, successful tumour dissemination is a balance between two opposing requirements:

sufficient mobility to leave the primary tumour but sufficient “immobility” to colonize secondary sites [30–32]. We postulate that eEF1A2 might make cells too migratory to successfully colonize secondary sites. Alternatively, eEF1A2 may enhance prognosis by altering other oncogenic process, perhaps tumour self-renewal potential or angiogenic capacity. Further investigation will be necessary to clarify the mechanism by which eEF1A2 leads to increased long-term survival.

eEF1A2 does not predict good survival probability in all cancers. For example, others have reported that eEF1A2 expression is a marker for poor survival in lung cancer [12]. This disparity in prognostic significance of eEF1A2 between ovary, breast and lung cancer implies that eEF1A2 is having markedly different biological effects in the three distinct tissues. An oncogene undertaking different roles and partaking in different signaling pathways often differs as it is expressed in different tumour types [33,34]. The reason why eEF1A2 might be highly expressed in one ovarian tumour sub-type remains unclear. The gene is not detectably expressed in normal mammalian ovary [2] so tumour-specific expression is unlikely to be related to any unique cell type in normal ovary. Similarly, the reason why eEF1A2 predicts long-term survival in only serous tumours is unknown. The origin and developmental history of ovarian tumours is not well characterized, since most tumours are identified only in more advanced stages. Thus, further investigation is necessary to determine the role for eEF1A2 in controlling the development of specific gynecological cancers.

Tomlinson et al. have previously reported that expression of eEF1A2 is associated with the clear cell histology, whereas we have found that high expression of eEF1A2 is only found in 8% of these tumour types as opposed to 75% of those they examined [35]. The discrepancies between the results can be attributed to two possible differences. Firstly, Tomlinson et al. examined eEF1A2 protein expression in a total of 44 tumours, only 5 of which were of the clear cell type. We have used 500 ovarian tumours, of which 132 were clear cell carcinomas. Secondly, Tomlinson et al. have grouped the tumours examined as either eEF1A2 negative or positive whereas the grouping used here is eEF1A2 negative to moderate expression and high expression of eEF1A2. Therefore, the different sample sizes, as well as the different method of categorizing the expression of eEF1A2 could explain the difference between our two studies.

In summary, we report here that eEF1A2 is highly expressed in ovarian tumours and enhances *in vitro* properties of ovarian cancer cells. Thus, eEF1A2 likely has some causal role in the ovarian neoplastic process. Consistent with this idea, we find that eEF1A2 expression predicts increased survival probability in serous ovarian cancer.

## Acknowledgments

We thank Sujeeve Jeganathan and Anne Morrow for their helpful discussions and critical readings of this manuscript. We also thank Lionel G. Filion for assistance with the flow cytometry.

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