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Expression of eEF1A2 is associated with clear cell histology in ovarian carcinomas: overexpression of the gene is not dependent on modifications at the EEF1A2 locus

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The tissue-specific translation elongation factor eEF1A2 is a potential oncogene that is overexpressed in human ovarian cancer. eEF1A2 is highly similar (98%) to the near-ubiquitously expressed eEF1A1 (formerly known as EF-1-α) making analysis with commercial antibodies difficult. We wanted to establish the expression pattern of eEF1A2 in ovarian cancer of defined histological subtypes at both the RNA and protein level, and to establish the mechanism for the overexpression of eEF1A2 in tumours. We show that while overexpression of eEF1A2 is seen at both the RNA and protein level in up to 75% of clear cell carcinomas, it occurs at a lower frequency in other histological subtypes. The copy number at the EEF1A2 locus does not correlate with expression level of the gene, no functional mutations were found, and the gene is unmethylated in both normal and tumour DNA, showing that overexpression is not dependent on genetic or epigenetic modifications at the EEF1A2 locus. We suggest that the cause of overexpression of eEF1A2 may be the inappropriate expression of a trans-acting factor. The oncogenicity of eEF1A2 may be related either to its role in protein synthesis or to potential non-canonical functions.

Keywords: ovarian tumour; clear cell carcinoma; eEF1A2; translation elongation

Ovarian cancer accounts for 4% of cases of female cancers; it has the highest fatality-to-case ratio of all gynaecological cancers, largely because the vast majority of cases are late-stage at presentation. Tumours arising from the surface epithelium represent the most common form of ovarian cancer. Although a number of molecular mechanisms underlying ovarian tumorigenesis have been identified, a single model of progression has not been described, possibly because of the heterogeneous nature of ovarian carcinoma (Shih Ie and Kurman, 2004). Primary ovarian adenocarcinomas are divided into four common distinct morphological subtypes: serous, mucinous, endometrioid, and clear cell. Although clear-cell tumours are frequently confined to the ovaries at presentation, they are associated with a poor prognosis and are thus treated as high-grade neoplasms (Schwartz et al, 2002). The incidence of clear-cell tumours among epithelial ovarian cancers is around 10%. Clear-cell tumours tend to be resistant to platinum-based chemotherapy, giving rise to a poor prognosis (Itamochi et al, 2002).

Translation factor eEF1A2 is a tissue-specific variant of eEF1A1 (previously called EF-1-α). While eEF1A1 is almost ubiquitously expressed, expression of eEF1A2 is normally confined to muscles and neurons (Lee et al, 1992; Knudsen et al, 1993). The gene encoding eEF1A1 is on 6q13 and eEF1A2 on 20q13.3 (Lund et al, 1996). The encoded proteins are 92% identical and 98% similar. Whereas lack of eEF1A2 gives rise to the wasted mouse phenotype (Chambers et al, 1998), which involves motor neuron degeneration (Newbery et al, 2005), inappropriate overexpression has now been linked to cancer. Anand et al (2002) showed that eEF1A2, while not normally expressed in ovary, is expressed in 30% of ovarian tumours. The mechanism for the overexpression appeared to be gene amplification in most, but not all, cases. Anand et al (2002) were also able to demonstrate that ectopic expression of eEF1A2 in NIH3T3 cells gives rise to colony formation in soft agar and increases growth rate. Furthermore, overexpression in rat fibroblasts enhances focus formation, and eEF1A2-expressing ES-2 ovarian cells and NIH3T3 cells injected into nude mice give rise to tumour formation. There was however no information on which types of ovarian tumours show overexpression of eEF1A2 and expression was assessed at the RNA level only. eEF1A2 has been identified in an expression microarray study as one of a number of genes that are highly expressed (ca. fourfold) in clear cell ovarian tumours than other histological subtypes of ovarian cancer (Schwartz et al, 2002). We recently showed that EEF1A2 is also a potential oncogene in breast tumours (Tomlinson et al, 2005).
We have now set out to examine eEF1A2 expression in a panel of ovarian tumours of defined histological subtypes. We have generated and used a panel of antibodies that allow us to distinguish between the highly related variants eEF1A1 (which is expressed in normal breast and ovary) and eEF1A2 (which is thought only to be expressed only in muscle and neurons under normal circumstances) (Newbery et al, in preparation). We show in a panel of ovarian tumours that while a high proportion of clear cell carcinomas overexpress eEF1A2, a far smaller proportion of serous, endometrioid and mucinous tumours have high levels of eEF1A2 expression. Furthermore, we show that (1) DNA copy number at the EEF1A2 locus is unrelated to expression level, (2) there are no activating mutations in the eEF1A2 coding sequence in tumours where there is overexpression in the absence of gene amplification and (3) the methylation status of the EEF1A2 gene is unrelated to expression level.

MATERIALS AND METHODS

Patient samples: ovarian tumours

Primary ovarian (HOV) tumour material and non-malignant tissues were obtained from patients having undergone gynaecological surgery in the Lothian University Hospitals NHS Trust. Institutional ethical approval was granted for this work by the Lothian University NHS Trust Medicine/Clinical Oncology Research Ethics Subcommittee. Tissue samples were excised and stored in liquid nitrogen. Non-malignant tissue samples were derived from patients who underwent bilateral oophorectomies for suspected malignancy, but were found to have benign histologies; samples were collected from apparently normal contralateral tumours. Tumours were reviewed by subspecialist gynaecological pathologists, and categorised according to stage and histological type and grade.

Quantitative real-time reverse transcription–PCR (RT–PCR)

RNA was prepared from ovarian samples and cell lines as described previously (Sellier et al, 2003). Total RNA was isolated from tumour and normal tissue using Qiagen RNeasy kits (Qiagen, Crawley, UK). RNA was treated with DNase using DNAfree kit (Ambion, Cambridgeshire, UK) and 1 μg was used for RT–PCR using Retroscript kit (Ambion). TaqMan Assay-on-Demand from Applied Biosystems, Cheshire, UK was used for RT–PCR manufacturer’s instructions (Applied Biosystems).

Western blots

Protein lysates from cell lines were prepared using previously published protocols (Gilmour et al, 2002); the same method was used for primary tumour samples, but in these cases tissue was initially homogenised in extraction buffer before determination of protein content. Western blot analyses using 10 μg protein were carried out using standard protocols. The blots were incubated with primary anti-eEF1A2 rabbit antibody diluted 1 : 200 in blocking solution, as well as primary anti-GAPDH polyclonal mouse antibody (Chemicon International, Hampshcir, UK) diluted 1 : 10000. Blots were then incubated in the appropriate HRP conjugated secondary antibody (Dako Cytomation, Cambridge-shire, UK) at 1 : 500. Detection was performed using enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry

A computerised search of the archives of the Department of Pathology was used to identify 168 cases of ovarian carcinoma of the common epithelial types. Slides were retrieved and the histological classification reviewed by a gynaecological pathologist. Four representative areas of viable tumour tissue were identified and marked on the slides, and the corresponding areas in the paraffin blocks used as the source of tissue for the tissue microarray (TMA). Tissue cores (0.6 mm diameter) were sampled from each of the four areas, and mounted into separate recipient paraffin blocks by the use of a custom-made instrument (Beecher Instruments, Silver Springs, MD, USA). In the ensuing paraffin array blocks, the tissue cylinders were aligned and marked for identification according to a chart. The recipient TMA blocks were baked at 56 °C for 10 min before sectioning, and 3 μm paraffin sections were cut by standard microtomy. A histoarray (CJ1) produced by SuperBioChips (AMS Biotechnology, Oxfordshire, UK) was also used. Formalin fixed, paraffin embedded, sections of human normal tissue, tumour tissue and TMAs were deparaffinised with xylene, rehydrated, treated with picric acid, and microwaved in citric acid at pH 6. Slides were blocked in a 1 : 5 dilution of sheep serum for 30 min at room temperature. Primary anti-eEF1A2 rabbit antibody was used at a concentration of 1 : 10 diluted in phosphate-buffered saline (PBS), for 40 min at room temperature and secondary goat anti-rabbit IgG biotin conjugated antibody (Dako Cytomation, Cambridgeshire, UK) was used at 1 : 200 at room temperature for 30 min. Slides were incubated with StreptABC complex/HRP (Dako Cytomation) at room temperature for 30 min and in diaminobenzidine (Sigma Fast DAB, Sigma, Dorset, UK) for 2 min at room temperature. Alternatively, after the primary antibody step slides were washed in PBS and three drops of ChemMate DAKO EnVision/HRP Rabbit/Mouse secondary antibody (Dako Cytomation) was added to each slide. Slides were incubated for 30 min at room temperature and then washed in PBS. The DAB-containing substrate working solution was prepared by mixing 50 parts ChemMate Substrate Buffer with 1 part ChemMate DAB+Chromogen (Dako Cytomation). One millilitre of this solution was added to each slide and incubated for 5 min. Finally, slides were counterstained in haematoxylin, dehydrated and mounted in plexigel.

Immunohistochemical scoring methods

The ovarian tumour histoarray (CJ1 SuperBioChips AMS Biotechnology, Oxfordshire, UK) and normal ovarian sections were given a histoscore. For each score, the percentages of the tumour tissue (excluding stroma) which stain strongly (3), moderately (2) and faintly (1) were assessed. The Histoscore was calculated by multiplying the percentage of tumour tissue staining by the score in each category, and adding these values to give a maximum of 300. Expression in the TMAs was assessed using a modification of the method of Tolia et al (2006).

Statistical methods

Fisher’s exact test was used to test for associations between positive protein expression and tumour subtypes of clear cell carcinomas vs all other tumour types combined.

Mutation analysis

For mutation analysis of the EEF1A2 gene, primer pairs were designed for each exon (see supplementary data). PCR was carried...
out using Tag polymerase from Invitrogen (Paisley, UK), except in the cases of exons 1 and 8, that required the use of DYNAzyme EXT DNA polymerase (Finzymes, NEB, Hitchin, UK) with cycling conditions consisting of an initial denaturation step at 94°C for 5 min followed by 32 cycles of 30 s at 94°C, 30 s at the annealing temperature and 30 s at 72°C (60 s when using DYNAzyme). PCR products were then sequenced using BigDye v3.1 (Applied Biosystems) according to the manufacturer’s conditions.

**Methylation analysis**

Methylation analysis was carried out using bisulphite sequencing of a 548 bp region of the EEF1A2 CpG island. The EZ DNA Methylation Kit (Zymo Research) was used to convert 1 μg of DNA from ovarian tumours HOV 104, 179, 548, and 557 and from normal whole ovary samples 440 and 470 according to the manufacturer’s protocol. The resulting DNA was amplified using the following primers: 5'-AGGGATTGGAATTAGTAGTTT and 5'-AAAAAAATCCACCTTAA and Roche Fast Start Taq DNA polymerase. Cycling conditions were a 5 min initial denaturation step at 95°C followed by 44 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 90 s. The converted PCR products were cloned into the pCR2.1 vector using the TA Cloning Kit (Invitrogen) following the manufacturer’s protocol. Clones (2–5 for each original DNA sample) were sequenced as before and the results analysed using BiQ Analyser software (http://biq-analyser.bioinf.mpi-inf.mpg.de/) (Bock et al, 2005).

**Quantitative real-time PCR analysis of copy number**

For EEF1A2 DNA copy number analysis two sets of intronic primers were designed (see supplementary data online). Primers designed to amplify microsatellite loci in regions of chromosomes, which are normally stable in ovarian cancers were used for normalisation of total DNA amount. This was based on the method of DNA copy number analysis used by Ginzinger normalisation of total DNA amount. This was based on the method which are normally stable in ovarian cancers were used for designing PCR primers. All primer sets were designed using the Primer3 software. 

For quantitative real-time PCR analysis we used a MyiQ Single Color Real-Time PCR machine (BioRad, Hemel Hempstead, UK) and iQ SYBR Green Supermix (2 ×) (BioRad). Standard curves were conducted on 100 ng DNA extracted from normal blood that was serially diluted five times from 1:10 to 1:100000. 200 ng of DNA from each tumour was used in a 25 μl reaction. Cycling conditions were an initial 8.5 min denaturing step at 95°C followed by 40 cycles of 95°C for 30 s, and 72°C for 30 s. Fluorescent signal collection was carried out at elongation (72°C). A melt curve was included to confirm primer specificity and minus DNA controls were included to confirm that there was no contamination. Standard curves were used to determine the PCR efficiency for each primer set. Primers were used at 200 nM giving a PCR efficiency of between 90 and 110%. EEF1A2 copy number was then calculated using the (Pfaffl (2001) method of analysis. The copy number of EEF1A2 in normal blood DNA was determined using an average value from analysis of five different blood DNA samples. The use of three microsatellite loci for normalisation allowed for the exclusion of outlying values. Copy number at the chromosome 20p microsatellites D20S804 and D20S819 was calculated using the standard curve method of analysis (Applied Biosystems User bulletin #3).

**RESULTS**

**Expression analysis of ovarian tumours**

We assessed RNA and protein samples taken from the same group of ovarian tumours, all of which had been assessed for histological subtype. Initially, we carried out real-time RT–PCR to assess the level of expression of EEF1A2 mRNA in the tumour panel. The results are shown in Figures 1A and B and Table 1; whereas three out of the four clear cell tumours analysed (75%) had detectable overexpression of EEF1A2, only four of the 18 serous tumours (22%), five of the 12 endometrioid tumours (42%) and neither of the mucinous tumours were overexpressing. Similar RT–PCR analysis of a panel of ovarian cancer cell lines shows most cell lines to overexpress EEF1A2 compared with a human leukaemic cell line (HL60), with levels as high as 70-fold higher in the most extreme instance (OVCA5). The results are shown in Figure 1C.

We went on to use anti-peptide antibodies (Newbery et al, in preparation) against EEF1A2. The antibodies were raised against synthetic peptides, which were designed to maximise the differences between eEF1A2 and all other tumour compared with the human leukemic cell line. We assessed RNA and protein samples taken from the same group of ovarian tumours, all of which had been assessed for histological subtype. Initially, we carried out real-time RT–PCR to assess the level of expression of EEF1A2 mRNA in the tumour panel. The results are shown in Figures 1A and B and Table 1; whereas three out of the four clear cell tumours analysed (75%) had detectable overexpression of EEF1A2, only four of the 18 serous tumours (22%), five of the 12 endometrioid tumours (42%) and neither of the mucinous tumours were overexpressing. Similar RT–PCR analysis of a panel of ovarian cancer cell lines shows most cell lines to overexpress EEF1A2 compared with a human leukaemic cell line (HL60), with levels as high as 70-fold higher in the most extreme instance (OVCA5). The results are shown in Figure 1C.

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was significant ($P = 0.03$, Fisher’s exact test). For the in-house array a less strong correlation with histological subtype was seen. Only one tumour fell into the ‘strong’ category, with a histoscore of 229, and this was a clear cell tumour, but there were far more negatively and weakly staining tumours overall; the total percentage of tumours with moderate or strong histoscores was 5% for this TMA in contrast to the 19% for the commercial TMA. Nevertheless, when the scores were combined, all three of the tumours with a histoscore of >200 were clear cell carcinomas.

### Table 1  eEF1A2 expression in ovarian tumours

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>RNA Number analysed</th>
<th>% positive</th>
<th>Protein-Western Number analysed</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear cell</td>
<td>4</td>
<td>75</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>Serous</td>
<td>18</td>
<td>22</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>12</td>
<td>42</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>Mucinous</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

GAPDH = glyceraldehydes-3-phosphate dehydrogenase. Samples are scored as positive at the RNA level if they have a score greater than 0.5 when normalised to GAPDH (see Figure 1A).

DNA analysis of the EEF1A2 locus in ovarian tumours

Anand et al (2002) have previously shown that the EEF1A2 locus is amplified in two out of three tumours they found to be overexpressing eEF1A2, suggesting that amplification of the gene may not be the only mechanism mediating overexpression. We did not have access to cell lines from the tumours we had studied at the RNA and Western level, but did have DNA, so we used a real-time PCR method to estimate the copy number of the EEF1A2 locus. This was based on the method of Ginzinger et al (2000), but control primers were selected from regions that are normally
genomically stable in ovarian cancer; three sets were used in case of between-tumour variation. The copy number of \textit{EEF1A2} was estimated using primers within an intron to avoid amplification of \textit{EEF1A1}. We also estimated copy number at two loci on 20p, to distinguish between amplification of 20q and polyploidy. The results are shown in Figure 4; \textit{EEF1A2} was amplified at a significant level (more than 2s.d. from normal DNA) in 12 of the 15 (80%) ovarian cancers expressing \textit{eEF1A2} at the RNA or protein level. Only one of these, tumour HOV12, also had an increase in copy number at 20p. However, all eight of those cancers not expressing \textit{eEF1A2} also had copy numbers significantly exceeding that found in normal diploid blood DNA. It is clear that there is no obvious correlation between copy number and gene expression; indeed, a non-expressing tumour, number HOV170, shows the greatest \textit{EEF1A2} copy number. The similar pattern of copy number changes between non-expressing and expressing tumours suggests that gene amplification is not the primary mechanism underlying the overexpression of \textit{EEF1A2}.

We therefore, went on to establish whether the methylation status of the \textit{EEF1A2} gene differs between ovarian tumours and normal ovarian tissue. We used bisulphite sequencing to compare the methylation profile of 548 bp of the CpG island at the 5' end of the \textit{EEF1A2} gene in DNA samples from normal ovary, from tumours that do not express \textit{eEF1A2} and from overexpressing.

![Figure 2](image-url) **Figure 2** (A) Western blot analysis of protein extracts from ovarian tumours. Sample numbers are the same as those shown in Figure 1A. The same blot is shown having been exposed sequentially to \textit{eEF1A2} and GAPDH antibodies with a stripping step in between each antibody. The controls are HeLa, which expresses both \textit{eEF1A1} and \textit{eEF1A2}, lymphoblastoid cells (LB) which express only \textit{eEF1A1}, and adult mouse muscle, which expresses only \textit{eEF1A2}. Clear-cell tumours are indicated by bold underlined type. (B) Western blot analysis of protein extracts from ovarian cancer cell lines. The negative control is a lymphoblastoid cell line and the positive controls are HeLa, which we have previously found to express \textit{eEF1A2} at high levels, and mouse muscle.

![Figure 3](image-url) **Figure 3** Immunohistochemistry of normal ovary (A, B) and ovarian cancer (C–F) sections. Staining in the normal ovary can be seen to be confined to luteinised stromal cells (N1) and low level expression in the ovarian surface epithelium (N2). HOV304 corresponds to one of the clear-cell tumors that was also analysed by RT–PCR and T1 and T2 are clear-cell tumours from the histoarray. T3 is a negative staining tumour. Magnification × 10.
tumours. It can be seen in Figure 5 that although the CpG dinucleotides immediately 5' to the CpG island were methylated, the CpG island itself was unmethylated in all cases, suggesting that hypomethylation of the EEF1A2 gene in tumours is not a mechanism for overexpression.

Finally, we sequenced the coding and untranslated regions of the EEF1A2 gene in those tumours, which were overexpressing EEF1A2, but which had low copy number (and were thus amenable to analysis by sequencing without the potential complication of sequence differences between gene copies). This group comprised

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**Table 2** Immunohistochemistry for EEF1A2 in different ovarian tumour types using a commercial TMA and an inhouse TMA

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Number analysed</th>
<th>Negative</th>
<th>%</th>
<th>Weak</th>
<th>%</th>
<th>Moderate</th>
<th>%</th>
<th>Strong</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Clear cell</td>
<td>35</td>
<td>19</td>
<td>54</td>
<td>10</td>
<td>29</td>
<td>3</td>
<td>9</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Serous</td>
<td>64</td>
<td>35</td>
<td>54</td>
<td>26</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>38</td>
<td>29</td>
<td>76</td>
<td>9</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mucinous</td>
<td>11</td>
<td>6</td>
<td>54</td>
<td>4</td>
<td>36</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>148</td>
<td>89</td>
<td>60</td>
<td>49</td>
<td>33</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

TMA = tissue microarray. A histoscore of 0 is recorded as negative, between 1 and 100 as weak, between 101 and 200 as moderate and over 201 as strong.

**Figure 4** Mean normalised EEF1A2 copy number in normal gDNA (horizontal stripes), ovarian cancers expressing EEF1A2 at the RNA and/or protein level (black bars), and ovarian cancers that do not express EEF1A2 (white bars). Error bars show 2s.d of the mean.

**Figure 5** 'Lollipop' diagrams produced by BiQ Analyser. Open circles denote unmethylated CpG dinucleotides, while closed circles denote methylated CpG dinucleotides. (A) Tumours 179 and 104, which do express EEF1A2 and show some methylation in CpG dinucleotides preceding the island, but no methylation within the CpG island. (B) Tumours 308 and 470 which do not express EEF1A2. There are very few methylated CpGs either within the island or preceding it. (C) DNA from two different normal whole ovaries. There is little methylation present at any CpG dinucleotide within the sequence.
We have carried out a number of analyses of ovarian tumour DNA to establish the mechanistic basis of the overexpression of eEF1A2. Although amplification of the gene is seen in many tumours there is no correlation between locus amplification and gene expression, suggesting that this cannot be the sole underlying mechanism of overexpression. Amplification was seen just as frequently in tumours that do not express eEF1A2; it would be of interest to examine the expression levels of other genes within this amplicon. We sequenced the EEf1A2 gene from a number of tumours with low copy number, but failed to find any mutations that could be activating or otherwise lead to overexpression (although it remains a possibility that there are mutations in regulatory regions of the gene in some tumours); similarly, there was no correlation between methylation status and expression. It is clear therefore that overexpression does not depend on genetic or epigenetic changes at the EEf1A2 locus; we suggest that the overexpression may be mediated by the inappropriate expression of a trans-acting factor in certain tumours. Microarray assays could be informative in this regard.

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