The RNA-binding protein CUG-BP1 increases survivin expression in oesophageal cancer cells through enhanced mRNA stability

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Survivin, a member of the IAP (inhibitor of apoptosis protein) family, plays important roles in maintaining cellular homeostasis and regulating cell-cycle progression. This IAP is overexpressed in oesophageal cancer cells, leading to uncontrolled cell growth and resistance to apoptosis. CUG-BP1 (CUG-binding protein 1) is an RNA-binding protein that regulates the stability and translational efficiency of target mRNAs. In the present paper, we report that CUG-BP1 is overexpressed in oesophageal cancer cell lines and human oesophageal cancer specimens. CUG-BP1 associates with the 3′-untranslated region of survivin mRNA, thereby stabilizing the transcript and elevating its expression in oesophageal cancer cells. Our results show that overexpression of CUG-BP1 in oesophageal epithelial cells results in increased survivin mRNA stability and consequently survivin protein expression. Conversely, silencing CUG-BP1 in oesophageal cancer cells destabilizes survivin mRNA, lowering the level of survivin protein. In addition, we have found that altering CUG-BP1 expression modulates susceptibility to chemotherapy-induced apoptosis. Overexpression of CUG-BP1 in oesophageal epithelial cells increases resistance to apoptosis, whereas silencing CUG-BP1 makes oesophageal cancer cells more susceptible to chemotherapy-induced apoptosis. Co-transfection experiments with small interfering RNA directed against survivin suggest that the anti-apoptotic role for CUG-BP1 is not entirely dependent on its effect on survivin expression.

Key words: apoptosis, CUG-binding protein 1 (CUG-BP1), mRNA stability, post-transcriptional regulation, survivin, 3′-untranslated region.

INTRODUCTION

The prevalence of oesophageal cancer in the United States has been increasing over the last 4 decades. The American Cancer Society estimates that there will be 16,980 new cases of oesophageal cancer and 14,710 deaths from this disease in 2011. Despite improvements in early detection and the improved efficacy of multimodality therapy in the treatment of this disease, overall 5-year-survival rates remain only 20% [1].

Oesophageal cancer cells demonstrate a marked resistance to apoptosis that is partly mediated by the overexpression of survivin, a member of the IAP (inhibitor of apoptosis protein) family [2]. Survivin is expressed during the G2/M phase of the cell cycle and associates with microtubules of the mitotic spindle. Overexpression of survivin functions to overcome this apoptotic checkpoint and favours the aberrant progression of transformed cells through mitosis [3]. In two separate studies examining squamous oesophageal cancer specimens, overexpression of survivin has been associated with poor prognosis [4,5]. Survivin overexpression has also been correlated with resistance to chemotherapy-induced apoptosis in oesophageal cancer specimens [6]. More recently, overexpression of survivin mRNA and protein have been observed in Barrett’s oesophagus compared with normal oesophageal epithelium, suggesting that survivin overexpression probably occurs relatively early in the metaplasia–dysplasia–invasive carcinoma sequence [7,8]. Importantly, a study of 59 patients found decreased overall survival in patients with elevated survivin expression in their resected oesophageal cancer specimens following induction of chemoradiation [9].

Current understanding of the mechanisms responsible for the regulation of survivin expression is incomplete. Transcription of the survivin gene is activated by a number of transcriptional factors including the β-catenin–TCF (transcription factor) complex, c-Myc and Stat-3 (signal transducer and activator of transcription 3) [10–12], and conversely inhibited by p53 [13,14]. However, little is known about the post-transcriptional regulation of survivin. CUG-BP1 (CUG-binding protein 1) is part of the CELF (CUG-BP1 and ETR3 like factors) family of RNA-binding proteins and its role in cancer is beginning to be elucidated. It was first discovered in myotonic dystrophy and was found to bind to abnormally extended CUG mRNA repeats [15]. The binding of CUG-BP1 to its mRNA targets has most often been associated with decreased translation and target mRNA degradation. Previous reports demonstrate that the binding of CUG-BP1 to the mRNA of both tumour necrosis factor-α and p21 results in enhanced target mRNA expression, through increased mRNA stability and translation respectively [16,17].

We have observed that CUG-BP1 is overexpressed in oesophageal cancer cells compared with oesophageal epithelial cells. Because survivin mRNA contains multiple potential CUG-BP1-binding sites, we hypothesized that CUG-BP1 bound to survivin mRNA and enhanced survivin protein expression. The experiments in the present study demonstrate for the first time that: (i) overexpression of CUG-BP1 is strongly correlated with the overexpression of survivin observed in oesophageal cancer cells; (ii) alteration of CUG-BP1 expression modulates susceptibility to chemotherapy-induced apoptosis; and (iii) co-transfection experiments with small interfering RNA directed against survivin suggest that the anti-apoptotic role for CUG-BP1 is not entirely dependent on its effect on survivin expression.

Abbreviations used: Ago2, Argonaute 2; CDK4, cyclin-dependent kinase 4; CR, coding region; CUG-BP1, CUG-binding protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, haematoxylin and eosin; HuR, Hu antigen R; IHC, immunohistochemical; IGF-1, insulin-like growth factor 1; IP, immunoprecipitation; Lsm4, Lsm4 homologue; U6 small nuclear RNA associated; P-body, processing body; qRT-PCR, quantitative real-time PCR; RNP, ribonucleoprotein; RT, reverse transcription; siRNA, small interfering RNA; UTR, untranslated region.

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cell lines and human specimens; (ii) CUG-BP1 binds to the 3′-UTR (untranslated region) of survivin mRNA; (iii) this interaction increases the half-life of survivin mRNA in oesophageal epithelial cells; and (iv) silencing CUG-BP1 in oesophageal cancer cells increases their susceptibility to chemotherapy-induced apoptosis.

EXPERIMENTAL

Cells and cell culture

The human oesophageal cancer cell lines TE7 and TE10 were received as gifts from Dr Nishihira (Tohoku University, Sendai, Japan). They were derived from an oesophageal adenocarcinoma and squamous cell carcinoma respectively, and characterized in the Cell Resource Center for Biomedical Research, Tohoku University, Tohoku, Japan. These cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, and 1% L-glutamine (Mediatech), and maintained in a 37°C incubator with 5% CO2 humidified air.

Human oesophageal epithelial cells (nhESO) were derived from oesophageal specimens harvested at the time of donor lung procurement. Approval was granted by the Washington University School of Medicine Institutional Review Board. As these tissues would have otherwise been discarded and the samples were de-identified, informed consent was not obtained. Oesophageal specimens were opened and washed with sterile medium. The mucosa was then removed by careful dissection along the submucosal plane separating the mucosal and muscular layers of the oesophagus. This mucosal layer was then cut into small pieces and digested in a collagenase medium to create a single cell suspension. These oesophageal mucosal cells were then maintained in BEBM (bronchial epithelial basal medium; Lonza) supplemented with 20% heat-inactivated fetal bovine serum and 1% L-glutamine (Mediatech), and maintained in a 37°C incubator with 5% CO2 humidified air.

Reagents and antibodies

Anti-human survivin antibody was purchased from R&D Systems. Anti-CUG-BP1, anti-caspase-3, anti-procaspase-3, anti-Lsm4 (LSM4 homologue, U6 small nuclear RNA associated), anti-Ago2 (Argonaute 2), and anti-CDK4 (cyclin-dependent kinase 4) antibodies were purchased from Santa Cruz Biotechnology. Anti-actin mouse monoclonal antibody was obtained from EMD Biosciences. (S)(+)-(++)-Camptothecin was purchased from Sigma–Aldrich.

Histological analysis of human oesophageal specimens

Representative sections were taken from both tumour and normal epithelium distant from the tumour from resected oesophagectomy specimens. The sections were deparaffinized in xylene and rehydrated in graded series of ethanol. Sections were pre-treated with heat-induced epitope retrieval using a pressure cooker and Target Retrieval Solution pH 6.1 (Dako), followed by endogenous peroxidase blocking for 5 min with 0.3% H2O2. The sections were then incubated overnight with rabbit anti-survivin or murine anti-CUG-BP1 antibodies at a 1:250 dilution at 4°C in a hydration chamber. Antibody detection was performed by incubation with biotinylated goat anti-rabbit or anti-mouse secondary antibody (Dako) respectively for 30 min at room temperature (25°C). Slides were developed for 5 min using dianaminobenzidine as the chromagen (Dako) and were counterstained with H&E (haematoxylin and eosin). As a negative control, tissue sections were reacted with non-immune rabbit IgG plus the secondary antibody.

Western blot analysis

Whole-cell lysates were prepared in buffer containing 2% SDS sample buffer [250 nM Tris/HCl (pH 6.8), 2% SDS, 20% glycerol and 5% 2-mercaptoethanol], and protein concentrations were determined using the BCA (bicinchoninic acid) Protein Assay kit (Pierce). Proteins (25 μg) were denatured in 5× SDS loading buffer at 95°C for 5 min, separated by SDS/PAGE on 10% or 15% gels (Bio-Rad Laboratories) and transferred on to nitrocellulose membranes. After 1 h of blocking with 5% non-fat milk, membranes were incubated with specific antibodies followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin, and signal was detected by Chemiluminescence Reagent (REN) and visualized by autoradiography. Densitometry analysis was performed via ImageJ software (http://rsbweb.nih.gov/ij/).

RNA interference

Cells were transfected with oligonucleotides targeting CUG-BP1, Lsm4 (Santa Cruz Biotechnology) or survivin (Cell Signaling Technology) or a control scrambled sequence (Qiagen) at a concentration of 300 pmol with Lipofectamine™ RNAiMAX (Invitrogen) for 6 h.

CUG-BP1 overexpression

Cells were transfected at 60–70% confluency in 60-mm plates with human CUG-BP, Elav-like family member 1 cDNA or the empty pCMV6-XL5 control vector (OriGene). CDNA (2 μg) was transfected into nhESO or TE7 cells with Lipofectamine™ 2000 (Invitrogen) for 6 h.

Preparation of synthetic RNA transcripts

cDNA from nhESO and TE7 cells were used as PCR templates for in vitro synthesis of biotinylated survivin CR (coding region) and 3′-UTR transcripts. The T7 RNA polymerase promoter sequence (5′-CAAGCTTCTAATACGACTCACTATAGGGAGA-3′) was appended to the 5′-end of all of the fragments. The primers used for the preparation of biotinylated transcripts spanning the CR and 3′-UTR are listed in Table 1.

RNA-protein binding assays

Biotinylated transcripts (6 μg) were incubated with 120 μg of cytoplasmic lysates for 1 h at room temperature. Paramagnetic streptavidin-conjugated Dynabeads (Dynal) were used to isolate the bound complexes, and Western blot analysis using antibodies recognizing CUG-BP1 was subsequently performed. To assess the association of both CUG-BP1 and Ago2 with endogenous survivin mRNA, mRNA IP (immunoprecipitation) assays were performed. Whole-cell lysates were used for IP in the presence of anti-CUG-BP1, anti-Ago2 or non-specific IgG. RNA in the IP materials was used in qRT-PCR (quantitative real-time PCR) reactions to detect the presence of survivin mRNA.

RT (reverse transcription)–PCR and qRT-PCR analysis

Total RNA isolation was carried out with the RNeasy mini kit (Qiagen) and reverse transcribed with the RT System (Promega). The resulting cDNA was amplified with primers
specific for survivin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The forward primer for survivin was 5'-TTTCTGCCACATCTCTGAG-3' and the reverse primer was 5'-TGTCGAGGAAGGCTTTCAGGT-3. The forward GAPDH primer was 5'-GTCAGTGGTGAGAACCTCGACT-3' and the reverse primer was 5'-AGGGTCTACAGGGCAACT-3'. The levels of GAPDH PCR product were assessed to monitor the even input of RNA in RT–PCR samples. qRT-PCR was performed using the 7500–Fast Real-Time PCR System with specific primers, probes and software (Applied Biosystems). The levels of survivin mRNA were quantified by qRT-PCR analysis and normalized to GAPDH levels.

Apoptosis assays
Cells were incubated with camptothecin and cell apoptosis was assessed after 6 h. Cells were photographed with a Nikon inverted microscope and quantified by morphological analysis. Approximately 75 and 300 cells were counted for nhESO and TE7 cells respectively, and the numbers of apoptotic cells were expressed as a fraction of the total number of cells counted. For caspase activation assays, cells were prepared in a similar manner and caspase-3 activity was assessed using the Caspase-3 Colorimetric assay (R&D Systems) according to the manufacturer’s instructions.

mRNA stability
mRNA stability assays were performed to measure the differences in mRNA half-lives. Briefly, pre-treated cells were incubated with actinomycin D (Sigma–Aldrich) for specified time points at a final concentration of 5 μg/μl and total RNA was extracted for PCR analyses.

Statistics
Analyses were performed using Prism 5.0 software (GraphPad). Data derived from multiple determinations were subjected to two-sided Student’s t tests and two-way ANOVA analyses. P values ≤0.05 were considered statistically significant.

RESULTS
CUG-BP1 and survivin levels are increased in human oesophageal cancer specimens and oesophageal cancer cell lines
Oesophagectomy specimens from patients with oesophageal adenocarcinoma containing both normal and malignant tissue were examined for both CUG-BP1 and survivin expression using IHC (immunohistochemical) staining. Figure 1(A), section a shows H&E staining of a representative adjacent benign squamous mucosa (upper panel) and oesophageal cancer specimen (lower panel) from the same patient. IHC staining for CUG-BP1 (Figure 1A, section b) depicts expression in the basal zone of the benign squamous mucosa with a loss of expression in the luminal surface. The adjacent malignant glandular tissue shows diffuse staining of CUG-BP1. Similarly, IHC staining for survivin (Figure 1A, section c) reveals high expression in the basal zone of the benign squamous mucosa, which is also reduced in the luminal surface. The adjacent malignant glandular tissue exhibits strong, diffuse staining for survivin.

Expression patterns of CUG-BP1 and survivin were also examined in nhESO cells and the TE7 and TE10 oesophageal cancer cell lines. Western blot analysis reveals that CUG-BP1 and survivin levels are significantly increased in both TE7 and TE10 cells in comparison with nhESO (Figure 1B). Densitometry analysis shows an approximate 1.3- and 1.75-fold increase in CUG-BP1 and a 1.6- and 2.2-fold increase in survivin in TE7 and TE10 cells respectively (Figure 1C). These findings indicate that the up-regulated expression of survivin is correlated with increased levels of CUG-BP1 in both human oesophageal cancer tissue samples and cell lines. This primary observation led us to speculate that the increased levels of CUG-BP1 in oesophageal cancer cells may play an important role in regulating survivin expression.

CUG-BP1 binds to the survivin mRNA 3'-UTR
We first examined the interaction of intracellular survivin mRNA with CUG-BP1 by immunoprecipitating CUG-BP1 under conditions which preserve its association with target mRNAs in RNP (ribonucleoprotein) complexes using an anti-CUG-BP1 antibody. Following isolation of RNA, the presence of survivin mRNA in these RNP complexes was confirmed by qRT-PCR analysis (Figure 2A). The association of survivin mRNA with CUG-BP1 was 8-fold higher in TE7 cells than nhESO cells in keeping with the increased levels of survivin in these cells. This interaction was specific, because survivin mRNA was almost undetectable in complexes isolated with control non-specific IgG antibodies.

On the basis of sequence analysis of survivin mRNA, we found multiple potential CUG-BP1-binding sites in both the CR and 3'-UTR. The formation of CUG-BP1–survivin mRNA complexes was tested by using biotinylated transcripts spanning either the CR or 3'-UTR regions of survivin mRNA. As shown in Figure 2B, section b, Western blot analyses of pull-down materials reveal that the survivin 3'-UTR transcript readily associates with CUG-BP1 in both nhESO and TE7 cells. These associations could be seen more prominently in TE7 cells, as expected because of previously observed increases in CUG-BP1 and survivin expression in TE7 cells. However, no complexes could be observed with the CR transcript, despite the presence of potential CUG-BP1-binding sites.

The survivin 3'-UTR was further subdivided into smaller transcripts in order to determine the specific binding location of CUG-BP1 (Figure 2C). Biotinylated transcripts spanning...
Figure 1  Differential expression of CUG-BP1 and survivin in normal oesophageal epithelial cells and oesophageal cancer cells

(A) A representative oesophagectomy specimen containing both normal and malignant tissue was examined after H&E staining and IHC staining for CUG-BP1 and survivin. Section a, representative photomicrograph (×100) of H&E-stained benign squamous mucosa (upper panel) and malignant and invasive glandular tissue (lower panel). Section b, IHC staining for CUB-GP1 (×100). The benign squamous mucosa shows expression in the basal zone (small arrowheads), with loss of expression at the luminal surface (large arrowhead). The adjacent malignant glandular tissue shows diffuse staining (arrow) of CUG-BP1. Section c, IHC staining for survivin (×100). The benign squamous mucosa shows marked staining for survivin in the basal zone (small arrowheads), which is reduced at the luminal surface (large arrowhead). The adjacent malignant glandular tissue shows strong diffuse staining for survivin (arrow). (B) Baseline levels of CUG-BP1 and survivin protein in nhESO cells and two oesophageal cancer cell lines, TE7 and TE10. Total protein (25 μg) was loaded into each lane, and immunoblots were probed with either a CUG-BP1- or survivin-specific antibody. Actin hybridization was performed as an internal control to ensure equal loading. The approximate sizes of CUG-BP1, survivin and actin are 60 kDa, 17 kDa and 42 kDa respectively. (C) Western blot analysis from (B) was quantified by densitometry and plotted relative to TE10 cells. The *P < 0.05 compared with nhESO cells. Results are means ± S.E.M.

eight fragments of the survivin 3′-UTR (each containing several potential CUG-BP1-binding sites) were constructed and analyses of pull-down materials were performed. CUG-BP1 was found to associate most strongly to F-2, moderately to F-1, F-5 and F-6, and less strongly to F-7 and F-8. CUG-BP1–survivin mRNA complexes were not observed in F-3 and F-4 even though several potential CUG-BP1-binding motifs were located in these regions (Figure 2D). Binding of survivin mRNA was specific to CUG-BP1, because no complexes were formed when transcripts were pulldown with actin. These findings clearly indicate that CUG-BP1 is able to bind and associate specifically with survivin 3′-UTR, and that its strongest affinity is to the region spanning the 912–1199 bp region.

CUG-BP1 manipulation leads to altered survivin mRNA stability and protein expression

Because basal levels of CUG-BP1 in nhESO cells are low, transfection of CUG-BP1 cDNA into these cells was performed in order to transiently elevate CUG-BP1 levels and assess the effects on survivin mRNA stability. As shown in Figure 3(A), a CUG-BP1-expressing vector was successfully transfected into nhESO cells, resulting in an approximately 3-fold increase in CUG-BP1 protein levels compared with the control vector. Importantly, this increase in CUG-BP1 protein levels was associated with an approximately 5-fold increase in survivin protein levels. Concurrently, overexpression of CUG-BP1 increased survivin mRNA levels (Figure 3B). This finding was due, in part, to stabilization of survivin mRNA as revealed by an increase in its half-life from approximately 3 h to 16 h (Figures 3C and 3D). These experiments reveal that overexpression of CUG-BP1 stabilizes survivin mRNA, resulting in elevated survivin protein levels in oesophageal epithelial cells.

In reciprocal experiments, siRNA (small interfering RNA) directed against CUG-BP1 (siCUG-BP1) was employed in order to reduce CUG-BP1 levels in TE7 and TE10 cells, because basal CUG-BP1 levels are elevated in these cell lines. At 48 h post-transfection, CUG-BP1 protein was effectively reduced approximately 3-fold compared with control siRNA (C-siRNA) transfection (Figure 3E). This reduction in CUG-BP1 expression resulted in decreased levels of survivin protein in TE7 and TE10 cells. To demonstrate that the down-regulation of survivin expression following CUG-BP1 silencing was not merely a reflection of global changes in cell growth, we examined the effect of silencing CUG-BP1 on CDK4. Western blot analysis shows that silencing CUG-BP1 increases CDK4 protein expression (Figure 3E, part c). Survivin mRNA was also decreased after silencing CUG-BP1 (Figure 3F). mRNA stability analysis of survivin was performed in TE7 cells, which reveals a decrease in the survivin mRNA half-life from approximately 45 h to 14 h (Figures 3G and 3H). These studies show that silencing CUG-BP1 destabilizes survivin mRNA, leading to decreased survivin protein expression in oesophageal cancer cells.

Binding of CUG-BP1 to survivin mRNA prevents its association with P-bodies (processing bodies)

To further determine the underlying mechanism by which CUG-BP1 stabilizes survivin mRNA, we examined the role of P-bodies in this system. P-bodies are intracellular organelles...
in which mRNA degradation occurs. We postulate that in the presence of CUG-BP1, survivin mRNA is prevented from being transported to the P-bodies, leading to its increased expression. To test this hypothesis, we measured the amount of survivin mRNA associated with Ago2, an important component of the P-body, following CUG-BP1 silencing. After silencing CUG-BP1 in TE7 cells, the detected levels of survivin mRNA were increased in the Ago2-immunoprecipitated materials compared with cells treated with C-siRNA. Survivin mRNA levels were minimal when immunoprecipitated with non-specific anti-IgG antibody (Figure 4A, section b). In separate experiments, we simultaneously silenced both CUG-BP1 and Lsm4, another important P-body component. As seen in Figure 4(B), silencing both CUG-BP1 and Lsm4 abrogates the reduction in survivin protein levels observed when CUG-BP1 alone is silenced. Together, these studies suggest that CUG-BP1 prevents transport of survivin mRNA to the P-body, thus maintaining high survivin expression levels in oesophageal cancer cells.

Overexpression of CUG-BP1 desensitizes oesophageal epithelial cells to apoptosis

As we have shown previously, the responsiveness of nhESO and TE7 cells to camptothecin-induced apoptosis correlates with survivin expression [2]. On the basis of our findings that
CUG-BP1 overexpression leads to increased survivin expression in nhESO cells, we hypothesized that overexpression of CUG-BP1 in nhESO cells would result in increased resistance to apoptosis. CUG-BP1-overexpressing nhESO cells were incubated with 1 μM camptothecin for 6 h followed by measurement of caspase-3 levels. Western blot analysis confirmed the successful overexpression of CUG-BP1 and correspondingly revealed a decrease in caspase-3 protein levels following exposure to camptothecin (Figure 5A). Apoptosis was also quantified by morphometry and the percentage of apoptotic cells was significantly decreased in cells overexpressing CUG-BP1 (Figure 5B). Furthermore, a caspase-3 colorimetric analysis was performed as another method to quantify apoptosis. Consistent with our previous findings, apoptosis was significantly reduced after the overexpression of CUG-BP1 compared with the vector control (Figure 5C). This series of experiments indicates that the overexpression of CUG-BP1 significantly enhances resistance of nhESO cells to camptothecin-induced apoptosis, possibly through increased survivin protein expression.

Silencing CUG-BP1 enhances susceptibility of TE7 cells to apoptosis

On the basis of the findings described above, we further hypothesized that silencing CUG-BP1 would increase the...
susceptibility of TE7 cells to apoptosis. Following successful CUG-BP1 silencing, TE7 cells were induced with 10 μM camptothecin for 6 h and apoptosis was measured. Western blot analysis confirmed the successful silencing of CUG-BP1 and correspondingly revealed an increase in caspase-3 protein levels following exposure to camptothecin (Figure 6A). Apoptotic cell counts, quantified by morphological changes associated with cell death, showed a 2-fold increase following CUG-BP1 silencing compared with C-siRNA transfection (Figure 6B). A caspase-3 colorimetric assay reveals an increased number of cells undergoing apoptotic cell death in siCUG-BP1-treated TE7 cells (Figure 6C). These findings indicate that silencing CUG-BP1 leads to increased susceptibility of oesophageal cancer cells to apoptosis, possibly through decreased survivin protein levels.

To test whether the increased susceptibility of TE7 cells to camptothecin-induced apoptosis observed following CUG-BP1 silencing was directly related to reduced survivin levels, a co-transfection experiment was performed. TE7 cells were transfected with both siRNA directed against survivin (siSurvivin) and the CUG-BP1 overexpression vector, or appropriate controls, and then subjected to camptothecin for 6 h. Western blot and densitometry analyses reveal successful silencing and overexpression of both survivin and CUG-BP1 respectively (Figure 7A). Despite the robust expression of CUG-BP1 in TE7 cells, CUG-BP1 levels approaching twice the baseline levels were achieved following overexpression. Apoptosis of uninduced and camptothecin-treated cells was visualized by pictomicrographs (Figure 7B) and quantified by cell counts (Figure 7C). In keeping with our previous results, TE7 cells treated with siSurvivin and control vector display a 3-fold increase in susceptibility to camptothecin-induced apoptosis compared with the double negative control (C-siRNA + vector). Interestingly, when CUG-BP1 was overexpressed in TE7 cells co-transfected with siSurvivin, these cells displayed a marked resistance to camptothecin-induced apoptosis, returning to baseline levels seen in the double negative control group. These important experiments suggest the anti-apoptotic effect exerted by CUG-BP1 is not entirely dependent on its effect on survivin expression.

**DISCUSSION**

Despite accumulating evidence detailing the importance of survivin overexpression in oesophageal cancer, the intracellular mechanisms regulating survivin expression are not clearly known [4–6,9]. The survivin gene has not been shown to be mutated in oesophageal cancer, suggesting critical roles for its transcriptional and post-transcriptional regulation. In the present study we describe the novel role of the RBP CUG-BP1 in survivin overexpression in oesophageal cancer cells. CUG-BP1 is overexpressed in oesophageal cancer cells and interacts specifically with the survivin mRNA 3'-UTR. This interaction results in enhanced survivin mRNA stability, leading to increased survivin protein expression.

To date, information on the post-transcriptional regulation of survivin has been scarce. We have previously shown that reducing survivin transcription in p53-null oesophageal cancer cells by overexpressing p53 does not decrease survivin protein
levels, suggesting an important role for post-transcriptional mechanisms in regulating survivin expression [2]. Vaira et al. [18] have described how activation of the IGF-1 (insulin-like growth factor 1)/mTOR (mammalian target of rapamycin) signalling pathway markedly increased survivin protein levels in serum-deprived prostate cancer cells. This increase in survivin protein was independent of increased survivin gene transcription or changes in protein stability. Instead, IGF-1 was found to modulate improved stability and enhanced translation of a pre-existing pool of survivin mRNA, although the mechanism by which this occurred was not elucidated. Finally, we recently showed that the RBP HuR (Hu antigen R) bound to the 3′-UTR of survivin mRNA and prolonged its half-life in oesophageal epithelial cells [19]. However, this interaction proved to be quite complex, as HuR overexpression in these cells also increased levels of p53, leading to decreased survivin transcription.

The results of the present study indicate that CUG-BP1 is overexpressed in both human oesophageal cancer specimens and oesophageal cancer cell lines compared with oesophageal epithelial cells. In human specimens, the expression patterns of CUG-BP1 and survivin are well correlated. In the normal squamous mucosa of the oesophagus, expression of both CUG-BP1 and survivin was localized to the regenerative zone of the submucosa with minimal expression in the epithelial layer. However, both CUG-BP1 and survivin expression were diffusely expressed throughout the oesophageal cancer tissue. Further studies are needed to elucidate the mechanisms whereby CUG-BP1 levels are increased in malignant tissue.

We found that CUG-BP1 binds to multiple sites in the 3′-UTR of survivin mRNA. Survivin mRNA contains several GU-rich and CUG repeats in both the 3′-UTR and CR. These sites have been recognized as target sequences for CUG-BP1 [20,21].

**Figure 5** Overexpression of CUG-BP1 enhances resistance to apoptosis in nhESO cells

(A) nhESO cells were transfected with either control vector (Vector) or the CUG-BP1 cDNA (CUG-BP1 O/E). Following transfection, cells were either not induced or exposed to camptothecin (1 μM) for 6 h and whole-cell lysates were obtained. Section a, Western blot analysis of uninduced (panel a) or camptothecin-induced (panel b) levels of CUG-BP1, procaspase-3, caspase-3 and actin. Section b, densitometry analysis quantifying caspase-3 protein levels relative to levels in uninduced samples. *P < 0.0001 compared with the vector control. (B) Apoptosis was measured in transfected cells as described in (A). Section a, representative morphological analysis of treated cells. Section b, percentage of apoptotic cells. *P < 0.01 compared with the vector control. (C) Early apoptosis was detected by caspase-3 colorimetric assay in cells prepared as described in (A). Whole-cell lysates were collected and lysed by lysis buffer, then incubated with appropriate reaction buffers, and the caspase-3 colorimetric substrate (DEVD-pNA) at 37 °C. Enzymatic activities for each condition were then read and recorded on a microplate reader using a 405 nm wavelength light after 1 h. *P < 0.05 compared with the vector control.
Post-transcriptional regulation of survivin by CUG-BP1

Figure 6  Down-regulation of CUG-BP1 in oesophageal cancer cells sensitizes cells to apoptosis

(A) Section a, representative immunoblots of CUG-BP1, procaspase-3, caspase-3 and actin in CUG-BP1-silenced TE7 cells following no induction (panel a) or exposure to camptothecin (10 \( \mu \)M) for 6 h (panel b). Section b, densitometry analysis of caspase-3 protein levels. *\( P < 0.0001 \) compared with C-siRNA. (B) Section a, representative morphological analysis of treated cells. Cells were prepared as described in (A). Section b, percentage of apoptotic cells. * \( P < 0.01 \) compared with C-siRNA. (C) A caspase-3 colorimetric assay was performed as described in Figure 5. * \( P < 0.05 \) compared with C-siRNA.

Interestingly, despite the presence of such sites in the CR of survivin mRNA, we were not able to demonstrate binding between the CR and CUG-BP1. Furthermore, experiments mapping CUG-BP1-binding activity in the survivin 3'-UTR demonstrated significant differences in binding affinity among the different fragments, although each fragment contained potential binding sites. One of the most interesting aspects of CUG-BP1 biology is that it can lead to accelerated decay of some targets while prolonging the half-life and enhancing translation of others. It is tempting to speculate that differences in the particular binding characteristics of CUG-BP1 to its target mRNA may account for these divergent effects.

The results reported in the present paper further show that overexpression of CUG-BP1 in oesophageal epithelial cells resulted in an increase in survivin mRNA half-life and protein expression. In reciprocal experiments, down-regulation of CUG-BP1 in oesophageal cancer cells was associated with a decrease in survivin mRNA half-life and decreased survivin protein expression. To determine if the relationship between CUG-BP1 and survivin was specific or related to a more global role of CUG-BP1 in regulating cell division, we examined the relationship between CUG-BP1 and CDK4 in TE7 cells. Previous data from our group showed that overexpressing CUG-BP1 leads to decreased translation of CDK4 mRNA and ultimately decreased CDK4 protein levels in rat intestinal crypt cells [22]. Similarly, in TE7 cells, silencing CUG-BP1 in TE7 cells was associated with an increase in CDK4 levels.

The precise mechanism by which CUG-BP1 contributes to increased survivin mRNA stability remains unknown. P-bodies function to repress expression of mRNAs delivered to these cytoplasmic structures [23,24]. Ago2 protein, a core component of the RNA-induced silencing complex, has been demonstrated to be an important component of the P-body and is involved in the repression of a number of mRNA transcripts [25–27]. We found that down-regulation of CUG-BP1 in oesophageal cancer cells is associated with an increase in the association of
survivin mRNA with Ago2 protein. In addition, we found that silencing Lsm4, an important activator of decapping in P-bodies, abrogates the reduction of survivin levels in TE7 cells following silencing of CUG-BP1 [28,29]. Taken together, these findings provide preliminary evidence to suggest that CUG-BP1 prevents the trafficking of survivin mRNA to the P-body. Further studies will be required to more fully characterize the mechanisms by which the binding of CUG-BP1 to survivin mRNA prevents the association of survivin mRNA with P-body proteins.

A key finding of the present study was the ability to modulate cellular responsiveness to chemotherapy-induced apoptosis by altering levels of CUG-BP1. CUG-BP1 overexpression in oesophageal epithelial cells was associated with an increased resistance to apoptosis. Conversely, silencing CUG-BP1 in oesophageal cancer cells increased the susceptibility of these cells to apoptosis. We have previously shown that the degree of responsiveness of nhESO cells and TE7 cells to camptothecin-induced apoptosis largely depends on the level of survivin expression [2]. The finding that altering CUG-BP1 expression directly correlated with survivin expression and cellular sensitivity to apoptosis suggested that CUG-BP1 mediates sensitivity to apoptosis by affecting survivin levels. However, by simultaneously overexpressing CUG-BP1 and silencing survivin in TE7 cells, we were able to completely abrogate the increased sensitivity to camptothecin-induced apoptosis seen only with survivin silencing. This finding raises the possibility that CUG-BP1 can enhance the expression of other anti-apoptotic proteins in addition to survivin. A recent report from Gareau et al. [30] describes the ability of CUG-BP1 to mediate resistance to the proteasome inhibitor bortezomid by stabilizing p21 mRNA and thereby increasing p21 levels. It is also possible that CUG-BP1 may destabilize transcripts of pro-apoptotic genes. We are currently exploring the broader role of CUG-BP1 in modulating apoptosis in oesophageal cancer cells.

**AUTHOR CONTRIBUTION**

Elizabeth Chang designed and conducted the experiments, analysed the data, drafted the paper and prepared the figures; James Donahue assisted in experimental design, performed experiments related to TE10 cells, analysed the data and prepared the paper; Lan Xiao, Yuhong Cui and Douglas Turner contributed to the discussion of the results; Jaladanki Rao and Jian-Ying Wang contributed to data analysis; William Twaddell performed and analysed immunohistochemical staining of oesophagectomy specimens; and Richard Battafarano supervised the experiments, assisted with the interpretation of data and managed the project.

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