HuR Regulates β-Tubulin Isotype Expression in Ovarian Cancer

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Abstract

The supply of oxygen and nutrients to solid tumors is inefficient because cancer tissues have an inadequate number of microvessels, thus inducing the selective growth of the most aggressive cancer cells. This explains why many of the factors underlying a poor prognosis are induced in hypoxic/hypoglycemic conditions. Among these factors, a prominent role in several solid tumors is played by the class III β-tubulin gene (TUBB3). The study described here reveals that glucose deprivation enhances TUBB3 expression at both the gene and protein levels in A2780 ovarian cancer cells. In silico analysis of TUBB3 mRNA sequence predicted a putative binding site for the RNA-binding protein Hu antigen (HuR) in the 3′ flanking untranslated region. A hypoglycemic-dependent engagement of this site was shown using RNA pull-down and ribonucleoimmuno-precipitation techniques. Thereafter, HuR gene silencing revealed that TUBB3 translation is HuR dependent in hypoglycemia because HuR silencing inhibited the entry of TUBB3 mRNA into cytoskeletal and free polysomes. Finally, the clinical value of this finding was assessed in a clinical cohort of 46 ovarian cancer patients in whom it was found that HuR cytoplasmic staining was associated with high levels of TUBB3 and poor survival. Cancer Res; 70(14): 5891–900. ©2010 AACR.

Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancies and, according to a recent meta-analysis of clinical studies, the 5-year overall survival rate of patients with advanced disease (stages III and IV) is around 20% (1). Because this disease is frequently only diagnosed in advanced stages, it is often impossible to remove the whole tumor mass at first surgery, making chemotherapy necessary. Unfortunately, one of the main factors underlying the high rate of mortality associated with ovarian cancer is resistance to chemotherapy. In fact, although 80% of patients respond to first-line chemotherapy, at relapse, they will be refractory to further treatment and will progress rapidly to a fatal outcome. Moreover, ~20% of patients do not respond to chemotherapy as a first-line treatment. The mechanisms underlying drug resistance are multifactorial. When analyzed in the context of their physiologic roles, the factors underlying drug resistance in ovarian cancer are related to survival programs stimulated by the microenvironmental conditions that are typical of a cancer that is characterized by hypoxia and shortage of nutrients (2). In fact, the oxygen and nutrient supply in solid tumors is often inefficient due to an inadequate number of microvessels in cancer tissues; unfortunately, this is sufficient to induce the selection of the most aggressive cancer cells in those tumors growing in the most hostile microenvironments. This explains why many of the factors underlying a poor prognosis are induced in hypoxic/hypoglycemic conditions.

Among these factors in ovarian cancer, a prominent role is played by the class III β-tubulin gene (TUBB3), and at the beginning its role was linked to resistance to paclitaxel (3, 4). Along with its specific role, later studies have shown that TUBB3 behaves as a pure prognostic factor for patients exhibiting the worst outcome (5) and a survival rate very close to that noted in subjects treated only palliatively (1). TUBB3 thereby represents a promising biomarker to identify ovarian cancer patients with aggressive disease and poor probability of benefit from the standard first-line platinum/taxane chemotherapy. Notably, similar trends have also been observed in other diseases, such as cancer of the lung and pancreas, and triple-negative breast cancer (6).

We have previously shown that TUBB3 expression is induced by hypoxia through a hypoxia-inducible factor-1α enhancement at the 3′ flanking region (7). Through this adaptation mechanism, cancer cells acquire the ability to...
better survive in hypoxic conditions. Hypoxia is frequently accompanied by a shortage of nutrients and glucose; thus, the aim of the present study is to determine whether hypoglycemia is an additional trigger for TUBB3 expression. It was found that hypoglycemia is indeed an additional factor in the expression of TUBB3. Furthermore, it was shown that TUBB3 regulation in hypoglycemic conditions involves the RNA-binding protein Hu antigen (HuR, embryonic lethal abnormal vision Drosophila-like 1), an RNA-binding protein that plays a pivotal role in the adaptive response to environmental stress, thereby identifying a potentially druggable step in the regulation of TUBB3 expression.

Materials and Methods

Cell cultures and reagents

A2780 human adenocarcinoma cell line was purchased from the European Collection of Cell Culture and grown in a fully humidified atmosphere of 5% CO2/95% air, in RPMI (Gibco) medium complemented with fetal bovine serum and antibiotics. Glucose-free RPMI (Gibco) medium was used for hypoglycemia experiments. Clonogenic assays were performed with or without drug treatment for a period of 72 hours. Cells were plated at a density of 320 cells/mL, and flasks were stained with Giemsa to count colonies (>50 cells) after an additional 14 days.

Actinomycin D (Sigma) was dissolved in distilled water and used at the final concentration of 5 μg/mL. Biotinylated-citidine triphosphate and T7 RNA polymerase were purchased from Invitrogen. Antibodies against HuR and TUBB3 protein were from Santa Cruz Biotechnology and Covance, respectively. Antibody against TUBB protein was generated in our laboratory, as previously described (8).

RNA silencing (siRNA) targeted to HuR (siHuR: 5′-AA-CAUGACCCAGAGAUGUAdTdT-3′) and negative control (siC: 5′-UACACCUGUAGCAGACCUUUdTdT-3′) not matching any human DNA sequence were synthesized by MWG. A2780 cells were transfected with oligonucleotide duplexes (100 nmol/L) that had been premixed with Transfectin (Bio-Rad) and incubated for 24 hours. All other chemicals were purchased from Sigma-Aldrich if not otherwise specified.

Quantitative reverse transcriptase PCR assay and ribonucleoprotein immunoprecipitation

Procedures to perform quantitative reverse transcriptase PCR (qRT-PCR) were previously described (8). Ribonucleoprotein immunoprecipitation assay was performed as described by Tenenbaum and colleagues (9). Briefly, cells were harvested by centrifugation and lysed in NT1 buffer [100 mM/L KCl, 5 mM/L MgCl2, 10 mM/L HEPES (pH 7.0), 0.5% Nonidet P40 (NP40), 1 mM/L dithiothreitol 100 units/mL RNase inhibitor (Roche), protease inhibitors (Sigma-Aldrich), and 0.2% vanadyl ribonucleoside complexes (New England Biolabs)]. Protein A/G plus agarose beads (Santa Cruz Biotechnology) were coated either with anti-HuR antibody or nonspecific IgG (Bio-Rad). Immunoprecipitation was set up in NT2 buffer [50 mM/L Tris-HCl (pH 7.4), 20 mM/L EDTA, 150 mM/L NaCl, 1 mM/L MgCl2, 0.05% NP40, and both RNase and protease inhibitors], and cell lysates were incubated with antibody-coated beads for 3 hours at 4°C. Beads were then washed extensively with NT2 buffer, and RNA was extracted with RNeasy plus kit (Qiagen), according to the manufacturer’s instructions. HuR-bound mRNA was then detected after reverse transcription with the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions and quantitative real-time PCR. Normalization of input mRNA was confirmed by qRT-PCR using equal amounts of starting cell lysates.

Biotinylated RNA probe pull-down assay

Probes were synthesized by PCR using the following primers: for TUBB3 3′ untranslated region (UTR) probe, forward primer 5′-TATTGCAGCTCCAGGCTGACG-3′ and reverse primer 5′-AAGGGTATCGACAGCAATGATT-3′; for TUBB 5′UTR probe (C probe), forward primer 5′-TCGCTTCAAGGTATGTATGAGT-3′; and reverse primer 5′-CTCAGGACGAGGACGAGGC-3′; for TUBB 3′UTR probe (A probe), forward primer 5′-CAGAATTTGTC-TTGTGCTGCCCT-3′ and reverse primer 5′-GTGGCAGGCACGTCTTCTAGA-3′; for TUBB3 UTR probe (B probe), forward primer 5′-TCTCTCCATTTTGGCAACATC-3′ and reverse primer 5′-GTGGCACGGACCTTGTCTAGA-3′. Negative control probes for TUBB3 and TUBB were obtained by PCR using the following primers: 5′-AGTGTC-TAAGCCCCGGAGCCAT-3′ (forward) and 5′-AGGTGGGGAGGACGAGGC-3′ (reverse) as for TUBB3 and 5′-TTCTTTACCCAAAAAAAAAAGAATGAC-3′ (forward) and 5′-GGAGAGGTTCCTCCCT-3′ (reverse) as for TUBB. PCR products were cloned in pGEM (Promega) under the T7 promoter and in vitro transcribed using T7 RNA polymerase (Invitrogen), in the presence of 14C-biotinylated cT (Invitrogen). After pull down with streptavidin beads, the proteins were run in SDS-PAGE and transferred to poly(vinylidene fluoride) membranes (Millipore), and Western blotting was done using anti-HuR antibody. Cytoplasmic extracts were precleared by centrifugation, and potassium acetate was added to a final concentration of 90 mM/L. After addition of RNase inhibitor (Roche; 100 units/mL) and yeast tRNA (20 μg/mL; Ambion), cytoplasmic extracts were precleared with streptavidin-conjugated agarose (Upstate) for 1 hour at 4°C with rotation. After centrifugation at 8,000 × g for 1 minute, the supernatants were mixed with in vitro transcribed biotinylated HuR probes or negative control probes, and the mixture was incubated for 1 hour at 4°C. Protein and biotinylated RNA complexes were recovered by addition of streptavidin-conjugated agarose at 4°C for 2 hours, with rotation. The precipitated complexes were extensively washed with binding buffer [10 mmol/L HEPES (pH 7.5), 90 mmol/L potassium acetate, 1.5 mmol/L MgCl2, 2.5 mmol/L dithiothreitol, 0.05% NP40, protease and phosphatase inhibitor cocktail, 0.5 mmol/L phenylmethylsulfonyl fluoride], boiled in SDS-PAGE sample buffer, and resolved by gel electrophoresis followed by Western blotting with anti-HuR antibody.
Generation of p-Decoy plasmid and stable transfection of A2780 cells

TUBB3 3′UTR probe was cloned in p-USE (+) plasmid (Upstate Biotechnology) to generate the p-Decoy vector. The construct was introduced in A2780 cells by electroporation and grown in selection with G418 antibiotic (Sigma) at the concentration of 1 mg/mL, in complete medium, using cells transformed with the empty vector (p-USE) as a control. After 3 weeks, G418-resistant cells were collected and checked for the integration of the construct through PCR.

Cell fractions and Western blotting assay

Cytoplasmic and nuclear extracts were prepared following procedures described by Lin and colleagues (10). Briefly, cells were resuspended in buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L dithiothreitol]. The cell suspension was kept on ice for 15 minutes, followed by vortexing for 10 seconds, after NP40 was added to a final concentration of 0.5% (v/v). After centrifugation at 10,000 × g for 30 seconds, the supernatant was collected as a cytoplasmic extract. The nuclear pellets were resuspended in buffer C [20 mmol/L HEPES (pH 7.9), 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol] and rocked on ice for 20 minutes. After centrifugation for 10 minutes, the supernatant was collected as a nuclear extract. The samples were then aliquoted and stored at −80°C for further assays.

Figure 1. A, bar chart of TUBB3 and TUBB mRNA expression (qPCR) in cells undergoing hypoglycemic stress for 24, 48, and 72 h. Bar and error bars refer to mean and SD of triplicate experiments, respectively. B, bar chart of TUBB3 and TUBB protein expression (Western blot) in cells undergoing hypoglycemic stress for 24, 48, and 72 h. Bar and error bars refer to mean and SD of triplicate experiments, respectively. C, representative Western blot of TUBB and TUBB3 expression in cells undergoing hypoglycemic stress for 24, 48, and 72 h. The total protein content was used as loading control. D, densitometric analysis of the band represented in C. Bar and error bars refer to mean and SD of three independent experiments, respectively.
Polysome fractionation was performed according to Hovland and colleagues (11). Briefly, cells were lysed in buffer I [10 mmol/L Tris-HCl (pH 7.6), 0.25 mol/L sucrose, 25 mmol/L KCl, 5 mmol/L MgCl₂, 0.5 mmol/L CaCl₂, 0.05% NP40], lysates were centrifuged at 1,000 × g for 5 minutes at 4°C, and the supernatant (free polysomal fraction) was collected. The pellet containing nuclei, cytoskeletal polysomal fraction, and insoluble membranes was washed again with buffer I, and cytoskeletal polysomes were extracted after incubation of the pellet in buffer II [10 mmol/L Tris-HCl (pH 7.6), 0.25 mol/L sucrose, 130 mmol/L KCl, 5 mmol/L MgCl₂, 0.5 mmol/L CaCl₂, 0.05% NP40] for 10 minutes. Lysates were centrifuged at 2,000 × g, and cytoskeleton/bound polysomes were obtained from the supernatants. After centrifugation at 3,000 × g, pellets were resuspended in buffer II with the addition of 0.5% NP40 to obtain membrane-bound polysomes. Every incubation was performed in the presence of both protease and RNase inhibitors. RNA from fractions was purified with RNAeasy plus RNA extraction kit (Qiagen), and 1 µg of RNA was reverse transcribed and subjected to qPCR analysis. The proteins were run by SDS-PAGE and transferred to poly(vinylidene fluoride) membranes for incubation with primary antibodies. After overnight incubation with primary antibodies in 5% nonfat milk in TBS plus 0.1% Tween 20, Western blots were incubated with horseradish peroxidase–conjugated secondary antibody (Bio-Rad), detection was performed using the enhanced chemiluminescence system (Amersham Bioscience), and images were acquired by Versadoc 2000 CDC camera (Bio-Rad). The quality of polysome fractionation was checked using the activity of lactate dehydrogenase (as marker of free polysomes) and the amount of vimentin (as a marker of cytoskeletal polysomes), and representative data are shown in Supplementary Fig. S1.

Immunohistochemistry

The expression of HuR was immunohistochemically assessed in 46 ovarian cancer cases admitted in the gynecologic oncology unit of our department and included in our previous study (5). Immunostaining was performed on 3-µm paraffin tissue sections mounted on poly-l-lysine–coated slides and dried at 37°C overnight. Immunohistochemistry was performed as previously described (5).
Results

A2780 ovarian cancer cells were cultured in hypoglycemic conditions for 24, 48, and 72 hours, and the expression of TUBB3 was monitored at both the gene and protein levels. At the mRNA level, TUBB3 expression increased by 7-fold (24 hours) to 12-fold (72 hours) compared with control levels (Fig. 1A). Levels of class I β-tubulin (TUBB) were also measured in parallel with class III β-tubulin (TUBB3). Hypoglycemia induced a decrease in the level of TUBB mRNA (0.4-fold), followed by a progressive restoration of levels (1- and 2.5-fold after 48 and 72 hours of hypoglycemia, respectively). Changes at the gene level were then compared in the same experiments with the magnitude of protein expression measured using Western blots, using the total protein content as measured with Coomassie blue as the loading control (Fig. 1B and C). TUBB3 protein expression progressively increased after 24, 48, and 72 hours of hypoglycemia, up to a 3-fold increase (Fig. 1D). Conversely, TUBB mRNA and protein levels slightly increased after 24 hours, but not after 48 and 72 hours, when a dramatic reduction was evident (Fig. 1B–D). These findings reveal that hypoglycemia, similarly to hypoxia, preferentially induces the expression of TUBB3 but not of the constitutively expressed TUBB.

This finding prompted us to screen the 5′ and 3′ flanking regions of the human TUBB3 gene in search of factors implicated in the regulation of RNA expression. From this in silico analysis, we identified a single putative region with the potential to engage HuR in the 3′ UTR of TUBB3 mRNA, a protein binding to AU-rich elements of mRNA. Analysis for the identification of putative HuR binding sites was extended to the other six tubulin isotypes. Three putative binding sites were also found for TUBB, one in the 5′ region (probe C) and two in the 3′ region (probes A and B), whereas no regions with the potential to engage HuR were found for the other β-tubulin isotypes. Specific RNA probes able to recognize these putative binding sites were designed for TUBB3 and TUBB, respectively (Fig. 2).

Biotinylated probes were incubated with protein extracts from normoglycemic and hypoglycemic A2780 cells, and pulled down with streptavidin-conjugated agarose beads. Western blot analysis of the pulled-down materials showed that all probes were able to capture HuR protein, whereas the negative control probes did not. When comparing the two conditions, probe B was unaffected by hypoglycemia, whereas probes A and C exhibited a divergent behavior, with a decrease and an increase in binding under hypoglycemic conditions, respectively. Regarding the TUBB3 probe, an increase in binding was found on hypoglycemic conditions (Fig. 2B). Taken together, these findings reveal that low glucose levels enhance HuR binding in 5′ and 3′ UTRs of the TUBB and TUBB3 mRNA, respectively. The in vivo association of HuR with endogenous mRNAs of the all the six β-tubulin isotypes was investigated through cytoplasmic HuR protein ribonucleoimmunoprecipitation followed by real-time quantitative PCR of the recovered mRNAs (Fig. 3). An anti-IgG antibody was used as a negative control in ribonucleoimmunoprecipitation. Results again showed that HuR engages both TUBB and TUBB3 RNAs, but not the other β-tubulin isotype messengers (Supplementary Fig. S2). However, there was a major difference between the binding to TUBB3 and TUBB in hypoglycemia: for TUBB, no modulation of the binding was detectable, whereas for TUBB3, there was an increased binding of HuR to TUBB3 mRNA in hypoglycemic conditions.

The functional impact of HuR in the regulation of TUBB3 was investigated using specific siRNAs to downregulate HuR expression (siHuR). A scrambled sequence not targeting known genes was used as a negative control (siC). A2780 cells were transiently transfected with siC and siHuR, and TUBB3 protein expression was monitored after 48 hours of hypoglycemia. siHuR was capable of inducing marked downregulation of HuR at both the mRNA (data not shown) and protein levels (Fig. 4A). Through this approach, we were able to show that HuR plays a pivotal role in inducing the expression of TUBB3 in hypoglycemic conditions because the increase in TUBB3 was no longer detectable following transfection with siHuR. In the same experiments, siHuR abrogated the effect of hypoglycemia on TUBB, thereby suggesting that HuR coordinates TUBB3 and TUBB expression.

Nuclear export of messengers into the cytoplasm is mediated by HuR in several genes. To assess a role, if any, for HuR in nuclear export of TUBB3 and TUBB mRNAs, we measured nuclear TUBB3 and TUBB mRNA levels in A2780 cells
transiently transfected with siC and siHuR. As depicted in Fig. 4B, levels of TUBB3 were consistently enhanced after downregulation of HuR expression, thereby suggesting that the specific transport of TUBB3 mRNA from the nucleus to the cytoplasm is hampered in the absence HuR. However, in hypoglycemic conditions, such nuclear/cytoplasmic export activity is not enhanced and thus the mechanism underlying the increased expression of TUBB3 in hypoglycemia may be different. On the other hand, the shuttling activity seems specific to TUBB3 because nuclear mRNA of TUBB and other genes such as RNAPOL2, HPRT, or GAPDH did not increase in siHuR-treated cells (Fig. 4B).

Because HuR has a key role in regulation of mRNA stability, de novo synthesis of mRNA was blocked with actinomycin D, and TUBB3 and TUBB mRNA levels were measured in the presence of siC and siHuR (Fig. 4C). In normoglycemia, siHuR treatment did not affect the mRNA levels of TUBB3, whereas a 2-fold increase was detected after glucose deprivation. This finding shows that HuR does not protect TUBB3 mRNA from degradation. Instead, we noticed the opposite phenomenon because TUBB3 levels were increased after siHuR treatment. Again, the sensitivity of TUBB mRNA and RNAPOL2 to degradation was not influenced by HuR in basal and hypoglycemic conditions (Fig. 4C). Instead, HPRT mRNA seems to be, at least partially, protected from degradation because siHuR treatment induced its downregulation regardless of the glucose concentration.

Figure 4. A, representative Western blot of HuR, TUBB, and TUBB3 protein expression in cells transfected with siC and siHuR with or without hypoglycemic stress. The total protein content was used as loading control. Bottom, densitometric analysis of the same gel. Bar and error bars refer to mean and SD of triplicate experiments, respectively. B, qPCR results showing the expression of TUBB3, TUBB, RNAPOL2, HPRT, and GAPDH nuclear mRNA in cells transfected with siC and siHuR with or without hypoglycemic stress. Bars and error bars refer to mean and SD of triplicate experiments, respectively. C, qPCR results of mRNA expression of TUBB3, TUBB, RNAPOL2, and HPRT in cells treated for 16 h with actinomycin D. D, qPCR results of mRNA expression of TUBB3, TUBB, RNAPOL2, HPRT, and GAPDH bound to free and cytoskeletal polysomes in cells transfected with siC and siHuR with or without hypoglycemic stress. Bars and error bars refer to mean and SD of triplicate experiments, respectively. Data are shown as fold induction with respect to the normoglycemic control. In siHuR cells, a dramatic reduction of the amount of mRNA bound to free and cytoskeletal polysomes is evident.
An additional level of HuR control in protein synthesis involves the ability to direct mRNA entry into polysomes to activate translation. To gain insights into this process, polysomes were collected in three fractions (cytoskeletal-bound, membrane-bound, and free). Using qPCR, mRNA levels were assessed in normoglycemic and hypoglycemic conditions, and after treatment with siC and siHuR. No detectable levels of TUBB3 mRNA were found in membrane-bound polysomes, whereas a dramatic decrease in siHuR was observed for TUBB3 mRNAs in free and cytoskeletal-bound polysomes (Fig. 4D). Other genes such as TUBB, RNAPOL2, HPRT, and GAPDH were not affected in free polysomes. On the other hand, in cytoskeletal polysomes, entry of HPRT and RNAPOL2 mRNA was enhanced in siHuR-treated cells (Fig. 4D). This finding supports the hypothesis that HuR enhances the preferential translation of TUBB3 mRNA in hypoglycemia through the selective entry of TUBB3 into polysomes at the expense of other mRNA in both free and cytoskeletal polysomes.

To further investigate the role of HuR protein in TUBB3 translation, the TUBB3-target sequence recognized by HuR was cloned in the multiple cloning site of the p-USE expression vector. A2780 cells were stably transfected with this

![Figure 5](https://www.aacrjournals.org/cancerres/70/14/5897/fig5.png)

**Figure 5.** A, Western blot reporting the expression levels of TUBB3, TUBB, and β-actin in control p-USE and p-Decoy cells. B, bar chart reporting dose-response curves obtained in clonogenic assays performed in p-USE and p-Decoy cells treated with cisplatin, paclitaxel, and thiocolchicine in control or hypoglycemic conditions. Colonies were counted 14 d after drug treatment. C, a representative experiment with cisplatin.
construct (thereafter referred to as p-Decoy). The empty p-USE vector was used as a negative control. Expression of TUBB3 and TUBB was assessed at the protein level in normoglycemic and hypoglycemic conditions. Similarly to gene silencing, also in this case, TUBB3 expression was reduced and not longer inducible in hypoglycemia (Fig. 5A). To have an idea of the functional impact of this phenomenon on drug sensitivity, clonogenic and growth inhibition assays were performed. No differences were detectable in growth inhibition assays when cells were cultured in the presence of cisplatin and paclitaxel for 72 hours (data not shown). In control cells (p-USE), hypoglycemia induced TUBB3 expression and cells displayed an increased resistance to cisplatin (Fig. 5B–C). This phenomenon was completely abrogated in p-Decoy cells, thereby demonstrating that functional inhibition of TUBB3 is accompanied by the reversal of drug resistance. Similar results were also obtained when paclitaxel and tiocolchicine were used (Fig. 5B).

The potential clinical value of our findings was investigated by examining HuR staining in a cohort of 46 ovarian cancer patients in whom we previously noticed the correlation between TUBB3 expression and overall survival (3). Strong staining was obtained in ovarian cancer tissue from all of the patients (46 of 46), but staining was confined to the cell nucleus in 36 of them, whereas the cytoplasm was also clearly positive in the remaining 10 patients. Representative cases are shown in Fig. 6A. When stratified for TUBB3 expression levels, HuR cytoplasmic staining was differentially represented with 1 of 18 and 9 of 28 patients who exhibited HuR cytoplasmic staining in low and high TUBB3 expression settings, respectively (Fig. 6B, $\chi^2 = 4.55, P < 0.05$). Moreover, patients with cytoplasmic staining exhibited a higher number of TUBB3+ cells (Fig. 6C), thereby suggesting that the mechanism we observed in vitro may contribute at least in part to the increase in TUBB3 expression in ovarian cancer patients with the worst outcome.

**Discussion**

The main clinical obstacle to the treatment of ovarian cancer and other solid tumors is drug resistance, and the main difficulty in overcoming this obstacle is the convergence of
several mechanisms to generate the drug-resistant phenotype. Among the different mechanisms, some are commonly expressed in the tumor tissue. For example, high TUBB3 expression seems to be related to a poor chemotherapy response and adverse prognosis in a wide setting of tumor tissues such as breast (12), stomach (13), pancreas (14), ovary (5), and lung (15). Notably, TUBB3 behaves mainly as a marker of innate resistance because it is not induced after chemotherapy response (5). This emphasizes the relevance of the link between the microenvironmental stimuli, which encourage selection of the most aggressive cancer cells, and the regulation of TUBB3, which is overexpressed at the expense of the constitutively expressed TUBB. We previously reported that hypoxia is a potent stimulus for TUBB3 expression (7). In the work presented here, we extend these findings and show that hypoglycemia, another hallmark of the tumor microenvironment (16), contributes to sustain the expression levels of TUBB3. Moreover, we obtained further insights into the posttranscriptional regulation of TUBB3 expression, which is associated with apparent discrepancies. When comparing the expression at the gene and protein levels, a strong correlation was found for TUBB3, but not for TUBB, because in hypoglycemic stress, we noticed a simultaneous posttranscriptional repression of TUBB. This discrepancy led us to hypothesize the presence of a concerted posttranscriptional mechanism of regulation between TUBB and TUBB3. Through an in silico analysis of those elements that are able to bind the regulatory regions of both genes, we identified HuR as a putative binding factor. In TUBB, HuR binding sites were detected at the 5′ and 3′ regions, whereas only a single site at the 3′ region was identified in TUBB3.

HuR is a RNA-binding protein that has been described as a main regulator for a series of factors involved in the control of apoptosis and survival programs (reviewed in ref. 17). This activity is exerted at several levels: control of nuclear/cytoplasmic export of specific mRNAs, protection of mRNA from degradation, and initiation of the translation through direct targeting into polysomes (reviewed in ref. 18). The contribution of each pathway was investigated using siRNA technology. TUBB3 mRNA is preferentially exported by HuR into the cytoplasm, but this mechanism cannot explain the increase in TUBB3 observed after glucose deprivation because there was no modulation in hypoglycemic stress. In addition, the increase in TUBB3 cannot be explained by the HuR-dependent control of RNA degradation. Indeed, there is a paradoxical enhancement of degradation associated with HuR activity, but this paradox is only apparent because for β-tubulin genes, translation is coupled to degradation through the unique mechanism of autoregulation (19). Therefore, the protection against mRNA degradation simply reflected the decrease in TUBB3 protein translation linked to HuR silencing. In fact, during hypoglycemic stress, HuR binding is selectively induced at the 5′ and 3′ regions of TUBB and TUBB3, respectively. HuR binding at the 3′ site specifically enhances the expression of several proteins involved in the adaptive response to toxic stimuli, an action that, for TUBB3, occurs mainly through direct targeting of the mRNA to free and cytoskeletal polysomes. Along with 3′ binding of TUBB3, HuR also engages the 5′ region of TUBB, but such binding leads to posttranscriptional repression. A similar phenomenon has been described for the insulin-like growth factor-1 receptor, whose expression is inhibited by HuR through binding at the 5′ flanking region (20). Thus, low levels of glucose induce a shift in the ability of HuR to bind to TUBB mRNA in the regulatory regions, with enhancement of binding at the 5′ site at the expense of that at the 3′ site. Through this mechanism, the final result is posttranscriptional repression of TUBB, coupled with the enhanced expression of TUBB3 mediated by the increased binding at the 3′ region. However, is this mechanism clinically relevant?

In ovarian cancer, we have shown that high levels of TUBB3 are linked to drug resistance and poor survival (5, 21). Here, we have shown that cytoplasmic HuR staining is maximally expressed in patients with high TUBB3 expression, thereby enforcing a previous finding reporting a correlation with poor survival of cytoplasmic HuR staining in ovarian cancer (22) and other diseases (23, 24). Because the HuR mechanism of TUBB3 mRNA targeting into polysomes occurs in the cytoplasm, these findings suggest that such posttranscriptional mechanisms are actually operating at the bedside and therefore could represent a novel druggable step in the fight against drug resistance.

To this end, results obtained in p-Decoy cells are encouraging. In fact, this approach led to a remarkable decrease of TUBB3 expression linked to the abrogation of the resistant phenotype for both paclitaxel and cisplatin, thereby proving that such mechanism could be druggable and potentially useful for those patients characterized by cytoplasmic HuR expression, high TUBB3 levels, and lack of response to platinum/taxane conventional chemotherapy.

In summary, during environmental stress, HuR orchestrates the specific translation of those mRNAs in the cytoplasm that are linked to survival programs, such as TUBB3. Therefore, inhibition of this binding could represent an attractive approach to disrupt these programs and restore drug sensitivity of the most aggressive ovarian cancer cells that were selected upon growth in hostile microenvironments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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