The mRNA-Destabilizing Protein Tristetraprolin Is Suppressed in Many Cancers, Altering Tumorigenic Phenotypes and Patient Prognosis

Sarah E. Brennan,1 Yuki Kuwano,2 Nadim Alkharouf,3 Perry J. Blackshear,4 Myriam Gorospe,2 and Gerald M. Wilson1

1Department of Biochemistry and Molecular Biology and Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine; 2Laboratory of Cellular and Molecular Biology, National Institute on Aging-NIH; 3Department of Computer and Information Sciences, Towson University, Baltimore, Maryland; and 4Laboratory of Signal Transduction, NIEHS-NIH, Research Triangle Park, North Carolina

Abstract

AU-rich element-binding proteins (ARE-BP) regulate the stability and/or translational efficiency of mRNAs containing cognate binding sites. Many targeted transcripts encode factors that control processes such as cell division, apoptosis, and angiogenesis, suggesting that dysregulated ARE-BP expression could dramatically influence oncogenic phenotypes. Using several approaches, we evaluated the expression of four well-characterized ARE-BPs across a variety of human neoplastic syndromes. AUFI, TIA-1, and HuR mRNAs were not systematically dysregulated in cancers; however, tristetraprolin mRNA levels were significantly decreased across many tumor types, including advanced cancers of the breast and prostate. Restoring tristetraprolin expression in an aggressive tumor cell line suppressed three key tumorigenic phenotypes: cell proliferation, resistance to proapoptotic stimuli, and expression of vascular endothelial growth factor mRNA. However, the cellular consequences of tristetraprolin expression varied across different cell models. Analyses of gene array data sets revealed that suppression of tristetraprolin expression is a negative prognostic indicator in breast cancer, because patients with low tumor tristetraprolin mRNA levels were more likely to present increased pathologic tumor grade, vascular endothelial growth factor expression, and mortality from recurrent disease. Collectively, these data establish that tristetraprolin expression is frequently suppressed in human cancers, which in turn can alter tumorigenic phenotypes that influence patient outcomes.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Gerald Wilson, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 North Greene Street, Baltimore, MD 21201. Phone: 410-706-8904; Fax: 410-706-8297; E-mail: gwils001@umaryland.edu.

©2009 American Association for Cancer Research.

Cancer Res 2009;69(12):5168–76

Introduction

The initiation and progression of cancer are exacerbated by dysregulated expression of proteins controlling diverse cellular phenotypes including differentiation, the cell cycle, apoptosis, angiogenesis, and cell invasiveness (1). Synthesis of these proteins is strongly influenced by the cytoplasmic concentrations of their respective mRNAs, which in turn depend on the kinetics of both mRNA synthesis and degradation. The cytoplasmatic stability of many mRNAs encoding oncoproteins, cytokines, and inflammatory mediators is controlled by AU-rich elements (ARE), a family of RNA sequences located within the 3′-untranslated region (3′-UTR) of each transcript (2, 3). ARE-directed control of mRNA decay is mediated, in part, through interactions with specific ARE-binding proteins (ARE-BP). For example, association of tristetraprolin, KH-type splicing regulatory protein, and some isoforms of AUFI accelerate decay of targeted transcripts, whereas some other proteins, such as HuR, protect mRNAs from degradation. By contrast, TIA-1 and TIAR do not appear to influence mRNA decay directly but rather suppress gene expression by inhibiting translation of associated mRNAs (reviewed in refs. 4, 5).

The presence of AREs in many mRNAs encoding cancer-related proteins, coupled with the role of ARE-BPs in regulating these transcripts, raises the possibility of links between the expression and/or activity of selected ARE-BPs and the initiation or progression of neoplasia. Recent studies have provided data supporting this hypothesis in selected experimental systems. For example, mice that constitutively overexpress the p37 isoform of AUFI (p37AUFI) develop sarcomas (6). By contrast, prostaglandin A2-dependent induction of p45AUFI in non-small cell lung cancer cells suggested an antiproliferative role for this protein, because cyclin D1 mRNA was destabilized concomitant with enhanced p45AUFI binding to the cyclin D1 mRNA 3′-UTR (7). Elevated HuR levels are associated with higher tumor grade in breast carcinomas (8), whereas ectopic overexpression of tristetraprolin in Ras-dependent tumor cells delayed tumor growth (9) and vascularization (10) in murine xenografts concomitant with destabilization of select target mRNAs. Finally, the translational regulatory protein TIA-1 was first identified as a proapoptotic factor in lymphocytes (11). Together, these examples suggest close and potentially complex relationships between ARE-BPs and cellular proliferation, angiogenesis, apoptosis, and even the stage of tumor development.

Although many mRNAs that encode tumorigenic products may be regulated by ARE-BPs, the prevalence of dysregulated ARE-BP expression and/or activity in the development of human cancers is largely unknown. Furthermore, mechanisms linking altered expression of specific ARE-BPs to neoplastic phenotypes remain poorly defined. To address these questions, we surveyed the expression of four well-characterized ARE-BPs across a wide range of tumor types using cDNA arrays and bioinformatic approaches. Although levels of each ARE-BP mRNA varied in selected cancers, expression of tristetraprolin was significantly repressed in the majority of solid tumors from diverse tissue sources. Tristetraprolin (also known as TIS11, ZFP36, and Nup475) is the prototype of a small family of RNA-binding proteins containing tandem CCCH zinc finger domains and recognizes ARE sequences through adjacent UAUU-binding sites (12, 13). Given our observations that tristetraprolin expression is diminished in many cancers, we predicted that decreasing tristetraprolin levels may enhance one or more tumorigenic phenotypes, thus giving cells that weakly express...
tristetraprolin a competitive growth or survival advantage. To test this model, we assayed the influence of tristetraprolin expression on three key tumorigenic phenotypes, cell proliferation, sensitivity to apoptotic stimuli, and expression of vascular endothelial growth factor (VEGF), in two different cultured cell models. Finally, we surveyed gene array data sets to determine whether loss of tristetraprolin correlated with tumor grade, enhanced expression of a known tristetraprolin substrate mRNA, and patient mortality. Together, these data show that tristetraprolin expression is widely suppressed in human cancers and that limiting cellular tristetraprolin levels can modulate several neoplastic phenotypes in a cell type-specific manner, which may contribute to increases in tumor grade and negative patient outcome in some tissues.

Materials and Methods

Cancer profiling cDNA array. A Cancer Profiling Array II (BD Biosciences) was probed for the expression AUF1, tristetraprolin, HuR, and TIA-1 mRNAs using radiolabeled cDNA fragments in ExpressHyb solution (Clontech) according to the manufacturer’s instructions. Hybridized $^{32}$P-labeled DNA probes were quantified using a PhosphorImager (GE Biosciences). Sample loading on the array was standardized using a ubiquitin cDNA probe (BD Biosciences).
Cell culture and transfections. HeLa/Tet-Off cells (Clontech) were maintained at 37°C and 5% CO₂ in DMEM (Invitrogen) supplemented with 10% FCS (Atlanta Biologicals) and 100 µg/mL G148 (Cellgro). Plasmids pT2hyg-FLAG-TTPwt and pT2hyg-FLAG-TTP C147R were transfected using Superfect (Qiagen), and stably transformed HeLa cell clones were isolated by selection in 100 µg/mL hygromycin B (Roche). Doxycycline (Sigma) was maintained (2 µg/mL) during selection and subsequent clonal expansion to prevent any tristetraprolin-dependent effects on cell viability. Several dozen independent hygromycin-resistant lines were screened for doxycycline-regulated expression of FLAG-TTPwt (or C147R) by Western blot using anti-FLAG antibodies.

Murine embryonic fibroblast (MEF) cultures were derived from E14.5 embryos of tristetraprolin knockout mice (Zfp36−/−) and wild-type littermates (Zfp36+/+) as described previously (14) and were maintained in DMEM supplemented with 10% FCS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine (Cellgro). All experiments involving MEFs in this study were done before the 12th cell passage.

Proliferation and apoptosis sensitivity assays. HeLa or MEF cells were seeded in 96-well plates at 1,000 per well and then returned to the tissue culture incubator. When measuring proliferation rates, cells were counted using the DHL Cell Viability and Proliferation Assay Kit (Anaspec) according to the manufacturer’s instructions. Cell numbers were determined by comparison of background-corrected fluorescence to standard curves of fluorescence versus cell number for each cell type and were consistent with data obtained from trypan blue exclusion assays (data not shown). To measure the sensitivity of HeLa and MEF lines to proapoptotic stimuli, cells were similarly seeded in 96-well plates and allowed to grow for 24 h before adding varying concentrations of staurosporine or cisplatin. Twenty-four hours afterwards, surviving cells were counted as described above. The IC₅₀ for each apoptotic stimulus was resolved using a four-parameter logistic equation (PRISM version 3.03).

Measurement of VEGF mRNA decay kinetics. Cellular VEGF mRNA decay rates were measured using actinomycin D time-course assays. Briefly, transcription was inhibited by addition of actinomycin D (5 µg/mL; Calbiochem) to the culture medium, and total RNA was purified at selected times thereafter. Time courses were limited to 4 h to avoid complicating cellular mRNA decay pathways by actinomycin D-enhanced apoptosis (15). VEGF mRNA levels were measured at each time point by quantitative real-time reverse transcription-PCR (RT-PCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA as described in Supplementary Information. First-order decay constants (k) were solved by nonlinear regression (PRISM) of the percentage of VEGF mRNA remaining versus time of actinomycin D treatment. Resolved VEGF mRNA half-lives (t₁/₂ = ln 2/k) are based on the mean ± SD of n independent time course experiments where n ≥ 3 or the mean ± spread where n = 2. Ribonucleoprotein immunoprecipitations used to detect interactions between FLAG-TTP and cellular VEGF mRNA were adapted from previously described methods (16).

Data analysis and statistics. Comparisons of mRNA levels and decay kinetics, drug IC₅₀ values, and cell proliferation rates were done using the unpaired t test. Differences yielding P < 0.05 were considered significant, with the exception of gene array comparisons, where a threshold of P < 0.001 was employed. Correlation analyses used the Spearman nonparametric test, whereas Kaplan-Meier comparisons were done using the log-rank test with events limited to death from recurrent disease. For correlation and survival analyses, differences yielding P < 0.05 were considered significant.

Results
Tristetraprolin expression is significantly repressed in many human tumors and tumor cell lines. To determine whether dysregulated ARE-BP expression might contribute to human tumor development, we first probed a Cancer Profiling Array to compare levels of AUF1, TIA-1, tristetraprolin, and HuR mRNAs between tumors and peripheral nontransformed tissues of 154 patients representing 19 different tissues. A change of one log₂ unit

Figure 2. Repression of tristetraprolin expression in cancer cell lines and human tumors. A, tristetraprolin mRNA was measured in nine human cancer cell lines concomitantly with tissue samples on the Cancer Profiling Array (lung, breast, and cervix). Mean ± SD tristetraprolin hybridization signals from 10 nontransformed tissues normalized to ubiquitin. B, Western blots probed for tristetraprolin and β-actin proteins in whole-cell extracts from breast tumors and patient-matched nontransformed tissue from five patients: 1, invasive lobular, undefined grade; 2, invasive ductal and ductal carcinoma in situ, grade 3 (Nottingham); 3, poorly differentiated invasive carcinoma, grade 3; 4, infiltrating ductal carcinoma and ductal carcinoma in situ, grade 2; and 5, extensive ductal carcinoma in situ, undefined grade. C, gene array data sets were screened for differential tristetraprolin mRNA levels using Oncomine version 3. Median tristetraprolin mRNA levels are shown by solid lines within each box on distribution plots. Box upper and lower limits represent the 75th and 25th percentiles, respectively, whereas the extended lines indicate 10th and 90th percentiles. Analysis methods, statistical comparisons, and data set sources are included in Supplementary Table S2.
myeloma, where tristetraprolin mRNA was induced relative to prolin mRNA levels were similarly decreased in ovarian, cervical, and lung carcinomas relative to healthy lung tissues in three independent experiments are summarized (Fig. 2).}

The Cancer Profiling Array experiments showed that tristetraprolin mRNA was suppressed in many tumors and cancer cell lines. Because this method was limited to ≤10 patients for each tissue type, we used two further approaches to validate these findings across larger sample populations. First, gene array surveys using the Oncomine utility (18) identified numerous patient populations exhibiting significant differences in tristetraprolin mRNA levels (P < 0.001). For example, tristetraprolin expression was repressed in lung carcinomas relative to healthy lung tissues in three independent patient pools (Fig. 2C; Supplementary Table S2). Tristetraprolin mRNA levels were similarly decreased in ovarian, cervical, and liver tumors. Contrasting this trend was smoldering multiple myeloma, where tristetraprolin mRNA was induced relative to healthy bone marrow. Prostate cancer represented yet another case, where three separate studies showed significantly less tristetraprolin mRNA in metastases versus primary tumors, suggesting that suppression of tristetraprolin might not be linked solely to oncogenesis but also to the stage of tumor development. Our second approach to survey tumor-dependent changes in tristetraprolin mRNA levels used the Cancer Genome Anatomy Project database based on the frequency of Expressed Sequence Tag and Serial Analysis of Gene Expression hits across large sample pools (19). In libraries derived from cancerous tissues, tristetraprolin tags were recovered 40% to 50% less frequently than from normal tissues by both Expressed Sequence Tag and Serial Analysis of Gene Expression approaches (Supplementary Table S3), supporting our observation that tristetraprolin expression is frequently repressed in tumors.

Restoring tristetraprolin alters cell morphology and suppresses tumorigenic phenotypes in HeLa cells. Conceivably, decreasing tristetraprolin expression could influence many cellular functions depending on the population of tristetraprolin substrate mRNAs present. To identify tristetraprolin-responsive tumorigenic phenotypes, we constructed stably transfected HeLa/Tet-Off cell clones expressing FLAG-tagged tristetraprolin from a tetracycline-responsive cassette. Additional clones expressed the tristetraprolin mutant C147R; disrupting this Zn2+-coordinating residue within the COOH-terminal zinc finger abrogates RNA-binding activity (20). Three principal observations prompted use of the HeLa/Tet-Off model. First, HeLa cells are derived from a cervical adenocarcinoma, where tristetraprolin expression is frequently repressed (Figs. 1 and 2). Second, endogenous tristetraprolin mRNA is virtually undetectable in HeLa cells (Fig. 2A). Third, potential tristetraprolin-dependent influences on cell proliferation or survival necessitated tight regulation of transgene expression to prevent negative selection during clonal expansion.
Doxycycline potently inhibited expression of FLAG-TTPwt or FLAG-C147R proteins in HeLa/Tet-Off clones (Fig. 3A). However, removing doxycycline induced FLAG-TTP to levels consistent with normal cervical tissue. Restoring wild-type tristetraprolin in HeLa cells induced morphologic changes; cells became flattened, increased surface contact area with culture plates, and developed irregular membrane edges (Fig. 3B). These phenomena specifically accompanied expression of FLAG-TTPwt because morphology did not noticeably change on induction of the C147R mutant or in TTPwt clones when the transgene was repressed (+Dox).

Because accelerated cell growth is a fundamental characteristic of tumors (1), we measured the effects of tristetraprolin on cell proliferation. Following doxycycline removal, HeLa cells expressing FLAG-TTPwt proliferated ~40% slower than untransfected cells (Fig. 3C; P = 0.001). An independent HeLa/FLAG-TTPwt cell clone yielded identical results (data not shown). This effect required the RNA-binding activity of tristetraprolin, because FLAG-C147R did not influence cell proliferation. Another property of tumor cells is the ability to evade apoptosis (1). Whereas transient tristetraprolin overexpression induces apoptosis in some cultured cell models (21), restoring tristetraprolin to physiologic levels in HeLa/FLAG-TTPwt clones did not induce significant cell death (data not shown). However, to determine whether tristetraprolin could influence apoptotic pathways, we measured the sensitivity of these cultures to two different proapoptotic stimuli. Staurosporine is a general protein kinase inhibitor that activates mitochondrial-directed apoptosis (22). Comparing IC50 values between HeLa clonal lines indicates that cells expressing FLAG-TTPwt are 2- to 2.5-fold more sensitive to staurosporine than either untransfected cells or those expressing the C147R mutant (Fig. 3D; Supplementary Table S4; P = 0.002). Poly(ADP-ribose) polymerase cleavage analysis (23) verified that cell death following staurosporine treatment was apoptotic (Supplementary Fig. S2). However, tristetraprolin did not enhance sensitivity to all proapoptotic stimuli. Cisplatin, which induces apoptosis by covalently modifying and distorting DNA (24), killed all HeLa clones with equal efficacy whether functional tristetraprolin was expressed or not (Fig. 3D).

Tristetraprolin limits VEGF expression in HeLa cells by accelerating VEGF mRNA decay. Aggressive tumors frequently enhance production of proangiogenic factors, such as VEGF (1). Several sequences resembling the UUAUUUAUU high-affinity tristetraprolin-binding motif (13) are located within the 3′-UTR of VEGF mRNA (data not shown), and this transcript is targeted by tristetraprolin in Ras-transformed fibroblasts and glioma cells (10, 25). In our HeLa clones, expression of FLAG-TTPwt suppressed VEGF mRNA by 50% (Fig. 4A; P = 0.0004) relative to untransfected cells or those expressing C147R. To determine whether FLAG-TTPwt limits VEGF mRNA levels by accelerating its decay, we measured VEGF mRNA stability using actinomycin D time-course assays (Fig. 4B). These experiments revealed that VEGF mRNA decays more rapidly in HeLa cells expressing FLAG-TTPwt (t1/2 = 0.83 ± 0.11 h; n = 3) than in untransfected cells (t1/2 = 2.3 ± 1.1 h; n = 2; P = 0.0008) or cells expressing the C147R mutant (t1/2 = 2.6 ± 0.1 h; n = 2). Next, we used ribonucleoprotein immunoprecipitation assays to determine whether tristetraprolin binding accompanied accelerated VEGF mRNA turnover. Using both conventional RT-PCR (Fig. 4C) and quantitative real-time RT-PCR (Fig. 4D), VEGF mRNA was easily detected in ribonucleoprotein immunoprecipitations.
tristetraprolin mRNA is plotted as the x-axis in the figure. The y-axis represents the normalized array signal intensity.

Discussion

The mRNAs encoding many proteins contributing to oncogenesis and tumor progression contain ARE-like sequences within their 3'-UTRs and, as such, may be regulated post-translationally by one or more ARE-BPs. Although individual ARE-BPs have been linked to specific tumorigenic phenotypes in selected experimental systems (described above), it was unclear whether...

www.aacrjournals.org
5173

Figure 5. Correlation analyses of tristetraprolin expression versus pathologic features and clinical outcomes in breast cancer. Tristetraprolin mRNA levels were extracted from an array data set containing expression profiles for 251 human breast tumors. This data set (Gene Expression Omnibus accession no. GSE3494) is described in ref. 27 and includes the Elston-Ellis pathologic grade of each tumor (40) and patient mortality from recurrent breast cancer over the subsequent 13 years.

A, tristetraprolin expression correlates negatively with breast tumor grade (r = −0.431; P = 1.1 × 10−6). Black circles, grade 1 tumors; green circles, grade 2 tumors; red circles, grade 3; open circles, undefined grade. Dotted lines, 95% confidence intervals of the regression solution. C, Kaplan-Meier analyses of patient cohorts ranked by tumor tristetraprolin mRNA expression. P values indicate cohort comparisons with patients expressing the highest tristetraprolin mRNA levels (black line).

B, VEGF mRNA levels correlate negatively with tristetraprolin mRNA in breast tumors (r = −0.281; P = 5.9 × 10−5). Black circles, grade 1 tumors; green circles, grade 2 tumors; red circles, grade 3; open circles, undefined grade. Dotted lines, 95% confidence intervals of the regression solution.

Cellular consequences of tristetraprolin depletion are cell-type specific. To this point, our survey of tristetraprolin-responsive tumorigenic phenotypes was limited to highly transformed cells. To evaluate tristetraprolin involvement in these pathways using a nontransformed background, MEFs were isolated from tristetraprolin knockout mice (TTP−/−) and wild-type littermates (TTP+/+). A Western blot verified the absence of tristetraprolin in TTP−/− MEFs (Supplementary Fig. S3A). Unlike our observations in HeLa cells, tristetraprolin expression enhanced proliferation of MEFs 3-fold (Supplementary Fig. S3B; P < 0.0001). Similar results were observed using MEFs derived from independent TTP+/+ and TTP−/− litters (data not shown). Furthermore, tristetraprolin expression dramatically decreased the sensitivity of MEFs to staurosporine-induced apoptosis (Supplementary Fig. S3C; Supplementary Table S4; P < 0.0001), unlike the drug-sensitizing effect observed in HeLa cells. Cisplatin sensitivity experiments revealed that both TTP+/+ and TTP−/− MEFs were highly resistant (IC50 > 50 μmol/L) to this compound.

In contrast to the divergent effects of tristetraprolin on the proliferation and staurosporine sensitivity of MEFs versus HeLa cells, tristetraprolin suppressed VEGF expression similarly in both cell backgrounds (Supplementary Fig. S3). VEGF mRNA levels were 45% lower in TTP+/+ versus TTP−/− MEFs (n = 3; P = 0.027). Also, actinomycin D time-course assays revealed that VEGF mRNA was degraded more quickly in TTP−/− MEFs (t1/2 = 1.03 ± 0.08 h; n = 2) than TTP+/+ MEFs (t1/2 = 2.6 ± 0.1 h; n = 2; P = 0.003 versus TTP+/+), analogous to observations in HeLa cells.

Suppressed tristetraprolin expression is a negative prognostic indicator in breast cancer. If decreased tristetraprolin expression is associated with tumor progression or aggressiveness, we predicted that tumors with varying tristetraprolin expression levels would behave differently in the patient population. To test this, we surveyed tristetraprolin expression across a gene array data set derived from the excised breast tumors of 251 patients (27). Considering tristetraprolin mRNA levels for each patient as a function of tumor grade revealed a negative correlation between these parameters, with more advanced tumors typically showing the weakest tristetraprolin expression (Fig. 5A). A second prediction is that if tristetraprolin destabilizes VEGF mRNA, then VEGF expression should be enhanced in tumors that express tristetraprolin poorly. Plotting relative tristetraprolin and VEGF mRNA levels for all breast tumors indicated a modest but statistically significant negative correlation between these transcripts (Fig. 5B). However, similar analysis using a gene array data set from 89 prostate tumors (28) revealed no correlative relationship between these mRNAs (Supplementary Fig. S4), although tristetraprolin expression was potently suppressed in prostate metastases (Supplementary Table S2). This observation was another example where the consequences of tristetraprolin suppression in tumors varied with tissue type. Finally, we ranked all 251 breast tumors by tristetraprolin mRNA level and then divided them into three equally sized cohorts. Kaplan-Meier analyses of patient outcomes indicated that patients with intermediate or low tumor tristetraprolin mRNA levels were 2- to 3-fold more likely to die from recurrent breast cancer following tumorectomy than patients whose tumors strongly expressed tristetraprolin at excision (Fig. 5C). Coincident correlation of low tristetraprolin mRNA levels with higher tumor grade, elevated VEGF expression, and poor patient survival indicates that suppressed tristetraprolin expression in tumors may represent a negative prognostic indicator in breast cancer.

Discussion

The mRNAs encoding many proteins contributing to oncogenesis and tumor progression contain ARE-like sequences within their 3'-UTRs and, as such, may be regulated post-translationally by one or more ARE-BPs. Although individual ARE-BPs have been linked to specific tumorigenic phenotypes in selected experimental systems (described above), it was unclear whether...

Suppression of Tristetraprolin Expression in Cancer

from cells expressing FLAG-TTPwt but not from untransfected HeLa cells or those expressing the C147R mutant, indicating selective association of this transcript with FLAG-TTPwt.

A Western blot revealed the absence of tristetraprolin mRNA in TTP−/− MEFs (Supplementary Fig. S3A). Unlike our observations in HeLa cells, tristetraprolin expression enhanced proliferation of MEFs 3-fold (Supplementary Fig. S3B; P < 0.0001). Similar results were observed using MEFs derived from independent TTP+/+ and TTP−/− litters (data not shown). Furthermore, tristetraprolin expression dramatically decreased the sensitivity of MEFs to staurosporine-induced apoptosis (Supplementary Fig. S3C; Supplementary Table S4; P < 0.0001), unlike the drug-sensitizing effect observed in HeLa cells. Cisplatin sensitivity experiments revealed that both TTP+/+ and TTP−/− MEFs were highly resistant (IC50 > 50 μmol/L) to this compound.

In contrast to the divergent effects of tristetraprolin on the proliferation and staurosporine sensitivity of MEFs versus HeLa cells, tristetraprolin suppressed VEGF expression similarly in both cell backgrounds (Supplementary Fig. S3). VEGF mRNA levels were 45% lower in TTP+/+ versus TTP−/− MEFs (n = 3; P = 0.027). Also, actinomycin D time-course assays revealed that VEGF mRNA was degraded more quickly in TTP−/− MEFs (t1/2 = 1.03 ± 0.08 h; n = 2) than TTP+/+ MEFs (t1/2 = 2.6 ± 0.1 h; n = 2; P = 0.003 versus TTP+/+), analogous to observations in HeLa cells.

Suppressed tristetraprolin expression is a negative prognostic indicator in breast cancer. If decreased tristetraprolin expression is associated with tumor progression or aggressiveness, we predicted that tumors with varying tristetraprolin expression levels would behave differently in the patient population. To test this, we surveyed tristetraprolin expression across a gene array data set derived from the excised breast tumors of 251 patients (27). Considering tristetraprolin mRNA levels for each patient as a function of tumor grade revealed a negative correlation between these parameters, with more advanced tumors typically showing the weakest tristetraprolin expression (Fig. 5A). A second prediction is that if tristetraprolin destabilizes VEGF mRNA, then VEGF expression should be enhanced in tumors that express tristetraprolin poorly. Plotting relative tristetraprolin and VEGF mRNA levels for all breast tumors indicated a modest but statistically significant negative correlation between these transcripts (Fig. 5B). However, similar analysis using a gene array data set from 89 prostate tumors (28) revealed no correlative relationship between these mRNAs (Supplementary Fig. S4), although tristetraprolin expression was potently suppressed in prostate metastases (Supplementary Table S2). This observation was another example where the consequences of tristetraprolin suppression in tumors varied with tissue type. Finally, we ranked all 251 breast tumors by tristetraprolin mRNA level and then divided them into three equally sized cohorts. Kaplan-Meier analyses of patient outcomes indicated that patients with intermediate or low tumor tristetraprolin mRNA levels were 2- to 3-fold more likely to die from recurrent breast cancer following tumorectomy than patients whose tumors strongly expressed tristetraprolin at excision (Fig. 5C). Coincident correlation of low tristetraprolin mRNA levels with higher tumor grade, elevated VEGF expression, and poor patient survival indicates that suppressed tristetraprolin expression in tumors may represent a negative prognostic indicator in breast cancer.

Discussion

The mRNAs encoding many proteins contributing to oncogenesis and tumor progression contain ARE-like sequences within their 3'-UTRs and, as such, may be regulated post-translationally by one or more ARE-BPs. Although individual ARE-BPs have been linked to specific tumorigenic phenotypes in selected experimental systems (described above), it was unclear whether...
these mechanisms contribute significantly to clinical neoplastic syndromes. In this study, comparing mRNA levels for four ARE-BPs in tumors versus patient-matched nontransformed tissues presented examples where expression of each RNA-binding factor was dramatically induced or inhibited in cancerous cells. However, several lines of evidence indicated that the mRNA-destabilizing factor tristetraprolin is frequently and dramatically suppressed in solid tumors. First, tristetraprolin mRNA levels were substantially decreased (≥50%) in the majority of tumors versus patient-matched peripheral tissues across 154 patients (Fig. 1), a trend also observed at the protein level among a small cohort of breast tumors (Fig. 2B). Second, constitutive expression of tristetraprolin mRNA is very weak in many cancer cell lines relative to untransformed human tissues (Fig. 2A). Third, meta-analysis of gene array data sets revealed decreased tristetraprolin mRNA levels in a variety of tumors relative to healthy tissue (Supplementary Table S2). Finally, Expressed Sequence Tag and Serial Analysis of Gene Expression tags encoding tristetraprolin mRNA fragments were recovered at a significantly lower frequency from cancerous versus normal tissues (Supplementary Table S3).

In cells, tristetraprolin recognizes selected ARE-containing mRNAs and targets them for decay, which limits the expression of their encoded gene products (12). However, in tumor cells that suppress tristetraprolin, we predict that this ARE-containing mRNA population will be stabilized, leading to increases in their cytoplasmic concentrations (Fig. 6). It follows, therefore, that tristetraprolin suppression may enhance cellular tumorogenic phenotypes by overexpression of proteins encoded by this mRNA population. To identify tristetraprolin substrate mRNAs that might encode protumorigenic factors, we surveyed the ARED database (brp.kfshrc.edu.sa/ARED/; ref. 29) for mRNAs containing high-affinity tristetraprolin-binding sites in their 3′-UTRs. Conceivably, overexpression of even a subset of these proteins following tristetraprolin suppression could enhance tumorogenic phenotypes in a variety of ways (Fig. 6; Supplementary Table S5). For example, mRNAs encoding the cell cycle/growth regulatory proteins cyclin D1 and cyclin-dependent kinase 2 both contain high-affinity tristetraprolin-binding motifs in their 3′-UTRs, as do mRNAs encoding the antiapoptotic regulators AKT1 and BCL-2. Consistent with these possibilities, we observed that HeLa cells lacking tristetraprolin exhibited both increased proliferation rates and partial resistance to staurosporine-induced apoptosis (Fig. 3). Our HeLa cell model also revealed that tristetraprolin limits the expression of VEGF, which can limit the aggressiveness of many cancers by abrogating the potential of VEGF to promote tumor vascularization (30). Finally, high-affinity tristetraprolin-binding sites were also observed in mRNAs encoding factors that regulate cell motility or invasiveness, including urokinase plasminogen activator receptor and fibrillin-2, suggesting that loss of tristetraprolin may increase the production of these proteins.

Although the influence of tristetraprolin on HeLa cells is consistent with a classic tumor suppressor, the tumorigenic consequences of tristetraprolin suppression can vary widely in different cell/tissue types. For example, in MEFs, tristetraprolin enhanced proliferation and decreased sensitivity to staurosporine-induced apoptosis (Supplementary Fig. S3), directly opposing our observations in HeLa cells. Experiments in mast tumor cells presented a third outcome, whereby tristetraprolin did not alter cell proliferation in culture, but precluded tumor growth in murine xenografts (9). Conversely, inhibiting VEGF expression represents a more consistent tumor-suppressing function for tristetraprolin. Here, VEGF mRNA was destabilized by tristetraprolin in both HeLa (Fig. 6B) and MEF (Supplementary Fig. S3) cells, and VEGF mRNA levels correlated inversely with tristetraprolin mRNA across 251 breast tumors (Fig. 6B). Tristetraprolin also destabilizes VEGF mRNA in glioma cells (25) and Ras-transformed fibroblasts (10), inhibiting vascularization of tumor xenografts from the latter. However, the absence of any correlation between VEGF and tristetraprolin mRNAs in prostate tumors (Supplementary Fig. S4) suggests that even induction of VEGF expression is not a uniform consequence of tristetraprolin suppression. As an example of how tristetraprolin may modulate tumorigenic phenotypes in a cell type-specific manner, let us consider the differential effects of tristetraprolin on the staurosporine sensitivity of HeLa versus MEF cells. Staurosporine promotes mitochondria-mediated cell death signaling by several distinct routes, including mitochondrial recruitment of p53 and inhibition of protein kinase B/AKT signaling pathways (31, 32). Although the molecular basis for modulation of apoptosis by tristetraprolin is unknown, two mRNAs recently shown to bind tristetraprolin in macrophages...
(33) provide some intriguing mechanistic possibilities. One tristetraprolin-binding mRNA encodes RBBP6 (also called PACT or P2P-R), a protein that binds p53 and inhibits its proapoptotic activity (34). By binding and destabilizing RBBP6 mRNA, tristetraprolin should decrease expression of this factor, thus diminishing its ability to suppress p53-dependent apoptosis. In this case, tristetraprolin could enhance sensitivity to some proapoptotic stimuli as was observed in HeLa cells. Another mRNA targeted by tristetraprolin in macrophages encodes the transcription factor ST18 (33), which induces expression of many proapoptotic and proinflammatory genes in fibroblasts (35). By destabilizing ST18 mRNA, tristetraprolin should suppress expression of ST18-inducible genes, thus decreasing apoptotic sensitivity analogous to our observations in MEFs. Given the variety of complex and often interconnected pathways directing apoptosis, we propose three mechanisms that may account for differential apoptotic sensitivity of HeLa versus MEF cells in response to tristetraprolin. First, tristetraprolin can only regulate substrate mRNAs with which it is presented; hence, the unique transcriptional profile of each cell type will dictate the mRNA substrate pool subject to tristetraprolin activity. Considering the above examples, it is possible that the genes encoding RBBP6 and/or ST18 may be transcribed in only selected cell models. Second, tristetraprolin is but one of a diverse collection of ~20 ARE-BPs identified to date (4, 12, 36). Although many ARE-BPs present distinct mRNA substrate preferences (13, 16, 37, 38), significant overlap between binding determinants can permit competitive, additive, or redundant functional relationships between these proteins. Accordingly, although suppression of tristetraprolin may enhance RBBP6 and/or ST18 expression by stabilizing their mRNAs, it is also possible that loss of tristetraprolin may permit redundant or even more potent mRNA destabilizing activities access to one or both of these mRNAs in a cell-specific manner. Finally, the relative influences of various proapoptotic and antiapoptotic pathways on staurosporine-induced cell death are likely to be unique in each cell model. As such, suppression of RBBP6 or ST18 (or other tristetraprolin substrate mRNAs) may influence the apoptotic sensitivity of HeLa cells differently than MEFs. Considering these possibilities, we believe that rigorous identification of tristetraprolin substrate mRNA populations across different cell types will be required to delineate the specific posttranscriptional regulatory networks controlled by this factor.

The central findings of this work are that tristetraprolin expression is frequently repressed in human cancers and that diminution of functional tristetraprolin can modulate diverse tumorigenic phenotypes. In breast cancer, decreased tristetraprolin expression may be a negative prognostic indicator, because patients with low tumor tristetraprolin mRNA levels disproportionately presented more advanced tumors and increased risk of mortality from recurrent cancer. However, frequent suppression of tristetraprolin in tumors from many tissue types suggests that its utility as a prognostic indicator may be much more inclusive. Other data suggest that measuring tumor tristetraprolin expression may be diagnostically useful. For example, tristetraprolin-dependent effects on the sensitivity of HeLa and MEF cells to staurosporine indicates that some apoptotic signaling pathways may involve factors encoded by tristetraprolin-regulated mRNAs. Because radiation and many chemotherapeutic strategies function by promoting tumor cell apoptosis, it follows that altered target cell sensitivity to such stimuli resulting from tristetraprolin suppression may limit (or enhance) treatment efficacy. The tristetraprolin-related protein BRF1 has been linked to apoptosis in cultured head and neck squamous cell carcinoma lines, where cispalatin sensitivity varies inversely with BRF1 levels (39). Curiously, tristetraprolin did not influence the sensitivity of either HeLa or MEF cells to cispalatin, possibly reflecting yet another example of the cell type-specific and/or protein-specific influence of ARE-BPs on tumorigenic phenotypes.

Disclosure of Potential Conflicts of Interest

The authors have filed a patent application covering the measurement of tristetraprolin expression as an oncologic diagnostic and prognostic tool.

Acknowledgments

Received 11/6/08; revised 3/11/09; accepted 4/6/09; published OnlineFirst 6/2/09.

Grant support: American Cancer Society grant RSG-07-293-01-GMC and NIH grant R01 CA104248 (G.M. Wilson) and the Intramural Research Program of the National Institute on Aging-NIH (M. Gorohe and Y. Kuwano).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


The mRNA-Destabilizing Protein Tristetraprolin Is Suppressed in Many Cancers, Altering Tumorigenic Phenotypes and Patient Prognosis

Sarah E. Brennan, Yuki Kuwano, Nadim Alkharouf, et al.