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BASIC—ALIMENTARY TRACT

The mRNA Binding Proteins HuR and Tristetraprolin Regulate Cyclooxygenase 2 Expression During Colon Carcinogenesis

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Background & Aims: **During tumorigenesis, loss of rapid messenger RNA (mRNA) decay allows for overexpression of cancer-associated genes. The RNA-binding proteins Hu antigen R (HuR) and tristetraprolin (TTP) bind AU-rich elements in the 3′ untranslated region of many cancer-associated mRNAs and target them for stabilization or rapid decay, respectively. We examined the functions of HuR and TTP during colon tumorigenesis and their ability to regulate cyclooxygenase (COX-2), a mediator of prostaglandin synthesis that increases in the colon tumor microenvironment.** ***Methods:*** **We evaluated expression of HuR and TTP during colorectal tumorigenesis and in colon cancer cells and associated them with COX-2 expression. HuR and TTP-inducible cells were created to investigate HuR- and TTP-mediated regulation of COX-2.** ***Results:*** **In normal colon tissues, low levels of nuclear HuR and higher levels of TTP were observed. By contrast, increased HuR expression and cytoplasmic localization were observed in 76% of adenomas and 94% of adenocarcinomas, and TTP expression was lost in >75% of adenomas and adenocarcinomas. Similar results were obtained for HuR and TTP mRNA levels in normal and staged tumor samples. In both adenomas and adenocarcinomas, COX-2 overexpression was associated with increased HuR and decreased TTP ($P < .0001$); similar associations were observed in colon cancer cells. HuR overexpression in cells up-regulated COX-2 expression, whereas overexpression of TTP inhibited it; limited TTP expression antagonized HuR-mediated COX-2 overexpression.** ***Conclusions:*** **Increased expression of the mRNA stability factor HuR and loss of the decay factor TTP occurs during early stages of colorectal tumorigenesis. These changes promote COX-2 overexpression and could contribute to colon tumorigenesis.**

Colorectal cancer is the third most common cancer among adult Americans and accounts for approximately 10% of all cancer-related deaths. In colorectal tumors, various genetic alterations have been identified that promote the initiation and progression of tumorigenesis. As a consequence of these defects, activation of multiple signaling pathways leads to enhanced expression of many growth- and inflammation-associated immediate-early response genes. A critical point in controlling the expression of these factors in intestinal epithelium occurs through posttranscriptional mechanisms that promote rapid mRNA decay, and a majority of immediate-early gene transcripts are inherently unstable because of the presence of 3′-untranslated region (3′UTR) adenylate- and uridylate (AU)-rich elements (AREs) that target the mRNA for rapid decay.[1] However, dysregulation in ARE-mediated decay is observed in colon cancer cells and tumors,[2,3] indicating the functional significance of posttranscriptional regulation in carcinogenesis.[4]

AREs mediate their regulatory function through association with multiple RNA-binding proteins that display high affinity for AREs.[1] The best studied ARE-binding proteins can promote rapid mRNA decay, mRNA stabilization, and translational silencing.[1] The Hu antigen R (HuR) protein is a ubiquitously expressed member of the *ELAV*-like family of RNA-binding proteins. HuR can function in an mRNA stabilizing capacity; when overexpressed in cells, HuR stabilizes ARE-containing transcripts and promotes their translation.[5] Contrasting the effects of HuR, tristetraprolin ([TTP], ZFP36, TIS11) is a member of a small family of tandem Cys3His zinc finger proteins and promotes rapid decay of ARE-containing mRNAs.[6] The binding of TTP to AREs targets the mRNA for rapid degradation through exosome recruitment and association with mRNA decay enzymes.[7]

Cyclooxygenases (COX) are key enzymes in the production of prostaglandins, and overexpression of the inducible isoform COX-2 has been shown to occur at multiple stages of colon carcinogenesis allowing for elevated prostaglandin synthesis to occur in the tumor

Abbreviations used in this paper: 3′ UTR, 3′-untranslated region; ARE, adenylate- and uridylate-rich element; COX-2, cyclooxygenase-2; HuR, Hu antigen R; IRS, immunoreactivity score; TTP, tristetraprolin.

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microenvironment.[8] In normal cells, COX-2 expression levels are potently regulated through AREs present in its mRNA,[9] whereas, under conditions of neoplastic transformation, the ability of the COX-2 ARE to promote posttranscriptional regulation is compromised.[10] Our prior work has demonstrated the ability of HuR to promote the stability of COX-2 and other ARE-containing mRNAs.[2] These findings and others demonstrate increased expression of HuR to occur in a variety of human cancers, including colorectal tumors, and promote ARE-containing gene expression.[2,3] However, the status of TTP expression and its ability to promote ARE-mediated mRNA decay in colorectal cancer is not known. In this report, we demonstrate that elevated HuR expression occurs concomitant with loss of TTP expression at an early stage of colorectal tumorigenesis that is associated with increased COX-2 expression and defines the role these opposing RNA-binding proteins have in controlling COX-2 expression. These findings offer what we believe are new insights into the loss of posttranscriptional regulation allowing for enhanced expression of COX-2 and other cancer- and inflammation-associated genes in colorectal cancer.

Materials and Methods

Colorectal Tissue Specimens

Immunohistochemical analysis was performed on paraffin-embedded human tissue array samples obtained from 2 sources. The colorectal carcinoma progression tissue array (CHTN2003CRCprog) from the Cooperative Human Tissue Network (National Cancer Institute [NCI], Rockville, MD) contained 42 normal colorectal tissue samples derived from 14 cases of nonneoplastic colonic mucosa, 42 adenoma tissue samples derived from 14 cases of adenomatous polyps, and 42 adenocarcinoma tissue samples derived from 14 cases of primary colorectal adenocarcinomas classified by pTNM staging. The colon cancer tissue array CO801 (US Biomax, Rockville, MD) contained 40 tissue cores each of colorectal adenocarcinomas and matched or unmatched adjacent normal tissue graded by histology. TissueScan qPCR colon cancer arrays (HCRT501) classified by American Joint Committee on Cancer staging were obtained from Origene (Rockville, MD).

Immunohistochemistry

Detection of HuR, TTP, and COX-2 protein was performed on serial sectioned tissue arrays. The following antibodies were used for immunohistochemical staining: monoclonal 19F12 HuR antibody (Molecular Probes, Eugene, OR) at 160 ng/mL (1:1250), 2 polyclonal TTP antibodies (N-18 and G-20) used in combination (Santa Cruz Biotechnology, Santa Cruz, CA) each at 800 ng/mL (1:250), and polyclonal COX-2 antibody (160126; Cayman Chemical Company, Ann Arbor, MI) at 1250 ng/mL (1:400). Standard staining protocols were used. Briefly, slides were hydrated, and antigen retrieval was performed in citrate buffer in a steam bath for 30 minutes. All primary antibodies were incubated on slides for 18 hours at 4°C. After washing in TBST, slides were incubated in corresponding biotinylated secondary antibodies following the Vecta Stain ABC kit protocol (Vector Laboratories, Burlingame, CA). Immunohistochemistry was visualized using the DAB peroxidase substrate kit (Vector Laboratories) and counterstained with hematoxylin (Sigma–Aldrich, St. Louis, MO).

Immunoreactivity Scoring

Stained tissues were examined for intensity of staining using a method similar to that previously described.[11] The intensity of staining in tumor sections was evaluated independently by 2 blinded investigators (L.E.Y. and D.A.D.). For each tissue section, the percentage of positive cells was scored on a scale of 0 to 4 for the percentage of tissue stained: 0 (0% positive cells), 1 (<10%), 2 (11% to 50%), 3 (51% to 80%), or 4 (>80%). Staining intensity was scored on a scale of 0 to 3: 0, negative staining; 1, weak staining; 2, moderate staining; or 3, strong staining. The 2 scores were multiplied resulting in an immunoreactivity score (IRS) value ranging from 0 to 12. These scores were then grouped together in 1 of 2 IRS categories: low (IRS, 0–6) and high (IRS, 7–12).

Messenger RNA Analysis

Relative levels for HuR, TTP, and COX-2 messenger RNA (mRNA) in human colon cancer tissues were determined by real-time PCR (qPCR) using the TissueScan qPCR colon cancer array (Origene, Rockville, MD). qPCR was performed according to the manufacturer's guidelines using Taqman probes for TTP (ZFP36) and HuR (ELAVL1) purchased from Applied Biosystems (Foster City, CA) using the 7300 PCR Assay System (Applied Biosystems). qPCR for COX-2 was performed using SYBR green PCR master mix (Applied Biosystems) and primers for COX-2 sense, 5′-GTCACAAGATGGCAAAATGCTG-3′ and antisense, 5′-TAAGATAACACTGCAGTGGCTC-3′. β-actin amplification was used as a loading control. Fold change in mRNA expression levels for each individual sample were normalized to the cycle threshold (C_t) using the first normal sample (N1). Total RNA extracted from HuR and TTP-inducible HeLa cells was used for complementary DNA (cDNA) synthesis as previously described,[9] and COX-2 mRNA levels were detected using Taqman probes for COX-2 (PTGS2) and normalized to 18S ribosomal RNA (rRNA) levels.

Cell Culture, DNA Transfection, and Adenoviral Infection

Human colon cancer cell lines LoVo, HT-29, SKCO1, and CaCo2 were obtained from the American Type Culture Collection (ATCC; Manassas, VA); HCA7