An A13 Repeat within the 3'-Untranslated Region of Epidermal Growth Factor Receptor (EGFR) Is Frequently Mutated in Microsatellite Instability Colon Cancers and Is Associated with Increased EGFR Expression

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Abstract

Colorectal cancers (CRC) with microsatellite instability (MSI) have clinical, pathologic, genetic, and epigenetic features distinct from microsatellite-stable CRC. Examination of epidermal growth factor receptor (EGFR) mRNA and protein expression levels in a panel of colon cancer cell lines identified strong expression of EGFR in multiple cell lines with MSI. Although no relationship between EGFR overexpression and the length of a CA dinucleotide repeat in intron 1 was observed, a variant A13/A14 repeat sequence within the 3'-untranslated region of the EGFR gene was identified, which was mutated by either mononucleotide or dinucleotide adenosine deletions in 64% of MSI cell lines and 69% of MSI colon tumors. Using a Tet-Off system, we show that this mutation increases EGFR mRNA stability in colon cancer cells, providing a mechanistic basis for EGFR overexpression in MSI colon cancer cell lines. To determine whether this mutation is a driver or a bystander event in MSI colon cancer, we examined the effect of pharmacologic and molecular inhibition of EGFR in EGFR 3'-UTR mutant MSI cell lines. Cell lines with an EGFR 3'-UTR mutation and that were wild-type (WT) for downstream signaling mediators in the Ras/RAF and PIK3CA/PTEN pathways were sensitive to EGFR inhibition, whereas those harboring mutations in these signaling mediators were not. Furthermore, in cell lines WT for downstream signaling mediators, those with EGFR 3'-UTR mutations were more sensitive to EGFR inhibition than EGFR 3'-UTR WT cells, suggesting that this mutation provides a growth advantage to this subset of MSI colon tumors. [Cancer Res 2009;69(19):7811–8]

Introduction

The important role played by epidermal growth factor receptor (EGFR) in the progression of cancer is illustrated by the clinical efficacy of multiple small-molecule and biological agents that target and inhibit this receptor. In colon cancer, two antibody-based therapies, cetuximab and panitumumab, have recently been approved for the treatment of metastatic disease (1).

EGFR signaling stimulates cell proliferation, angiogenesis, and metastatic spread and inhibits apoptosis of colon cancer cells (2–4). Activation of EGFR is initiated by binding of one of several ligands [EGF, transforming growth factor (TGF), or amphiregulin], which leads to EGFR phosphorylation and oligodimerization. EGFR phosphorylation in turn activates the Ras/Raf/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)-Akt, and signal transducer and activator of transcription pathways (3, 5).

EGFR overexpression has been reported to occur in 30% to 85% of human colon tumors (3, 5–8), and some, although not all studies, have linked EGFR expression levels with clinical outcome (7, 9). Mechanisms by which EGFR overexpression occurs in colon cancers are mediated by polysomy of chromosome 7 or on rare occasions (<1%) by EGFR gene amplification (8). The length of a polymorphic CA repeat element present in intron 1 has also been shown to regulate EGFR levels (10).

In the present study, we observed frequent overexpression of EGFR in colon cancer cell lines with microsatellite instability (MSI). Colon cancer can be broadly classified as microsatellite stable (MSS) or as having MSI (11). Although deregulation of β-catenin–T-cell factor signaling is the primary event driving both of these forms of colorectal cancer (CRC), several mutational, cytogenetic, and epigenetic differences exist between MSS and MSI tumors. These include the propensity for MSS tumors to be aneuploid, whereas MSI tumors are largely diploid, and a higher frequency for methylation-mediated tumor suppressor inactivation in MSI tumors (11–13). Differences in histopathologic presentation and prognosis between these two groups have been clearly defined (14–16), whereas differences in response to 5-fluorouracil–based adjuvant chemotherapy have been suggested (17). MSI colon cancers can be further separated into familial (hereditary non-polyposis colorectal cancer) or sporadic MSI. Patients with familial MSI colon cancer inherit mutations in one of several DNA mismatch repair genes, although mutations in MLH1 and MSH2
are most common (11, 16). Tumors arise in these patients typically in the 4th decade of life following loss of heterozygosity (11). In comparison, sporadic MSI is driven largely by epigenetic silencing of the MLH1 locus with significantly later tumor onset (18). Mutagenic repetitive elements are particularly prone to mutations, and truncating mutations due to mutations in repeat elements within the coding sequences of TGF-βRII (19) and Bax (20) are frequently observed in MSI colon tumors. In the present study, we identify a novel deletion mutation in a polyA(13) repeat element within the 3'-untranslated region (3'-UTR) of EGFR in a high percentage of MSI colon cancers, which is linked to EGFR overexpression. Functional studies show that this mutation enhances EGFR mRNA stability. Importantly, we show that this mutation provides a growth advantage in MSI colon cancer cell lines that are devoid of activating mutations in downstream signaling mediators.

Materials and Methods

Cell lines and cell culture. The source of all cell lines has previously been described (21). The hypomorphic Dicer knockout cell line (Dld-1 Dicer−/−) and parental line were generously provided by the Vogelstein/Kinzler laboratory (22). All cells were maintained in modified minimal medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1% HEPES, 1% nonessential amino acids, and 1% antibiotics and were grown at 37°C. For actinomycin D assays, cells were treated with 5 μM actinomycin D (Sigma). For determining sensitivity to cetuximab, cells were treated with cetuximab (20 μg/mL) for 24 h. Effects on cell growth were determined in vitro by propidium iodide staining and fluorescence-activated cell sorting analysis as previously described (23).

Isolation of RNA and reverse transcription-PCR. The methods of RNA extraction, reverse transcription, and real-time PCR were as previously described (23). Primers were used as follows: EGFR, ATGCTTCAAACCC-CACAC (forward) and GCCCTTGCATTCTCAC (reverse); Dicer, AATAATGGGCACTTGATGAC (forward) and GAATGTTGGTGGG-TAGCA (reverse); p21, ATGTGCTCTTGTCCTGGTTTC (forward) and CATTGGTGGAGGCTTGGTA (reverse); and actin, CACCTTCACCGTCGCTT (forward) and GATGAGATTTGGACGTCTT (reverse).

Western blot. Western blot analysis was performed as previously described (23). Anti-EGFR was obtained from Cell Signaling and anti-Dicer was obtained from Abcam.

Determination of 3'-UTR polyA and CA repeat length by direct sequencing. Genomic DNA extracted from colon cancer cell lines and from fresh-frozen resected primary colorectal tumors was PCR amplified with PCR supermix (Invitrogen). For amplification of the polyA repeat within the EGFR 3'-UTR, the following primers were used: TACAGAAACGCATCCAG-GCAT (forward) and GCCCTTGGGAGGAGCTGTGA (reverse); and actin, CACCTTCACCGTTC-GAG (forward) and GATGAGATTTGGACGTCTT (reverse).

Determination of EGFR 3'-UTR polyA and intron 1 CA repeat length by fragment analysis. The same forward primers used in the direct sequencing of the 3'-UTR polyA repeat and first intron CA repeat were used with the exception that the FAM fluorescence dye (Applied Biosystems) was conjugated to the forward primer for the 3'-UTR polyA repeat analysis and the VIC fluorescence dye was conjugated to the forward primer for the CA repeat analysis. Reverse primers were also the same with the exception of addition of the 7mer Fragment Analysis Research Group consensus sequence to the 5' end. PCR amplification was performed using AmpliTaq Gold (Applied Biosystems). Fragment analysis was performed at the DNA Sequencing Facility at Albert Einstein College of Medicine.

Construction of tetracycline-regulated luciferase-EGFR 3'-UTR reporter construct. The entire 1,720-bp 3'-UTR of EGFR was PCR amplified with the primers CCACGGAGGATGATGGAG (forward) and AGAGTGGAATTGAATATGGTTTTAT (reverse) using AmpliTaq Gold. The purified PCR fragment was subcloned downstream of the luciferase gene in pGEM-Luc (Promega) linearized by Stul (New England Biolabs). Due to PCR-introduced error, clones containing A12 and A13 repeats were also identified at this time and likewise cloned downstream of the luciferase gene in pGEM-Luc. The luciferase-EGFR 3'-UTR fragment containing either an A11, A12, or A13 [wild-type (WT)] repeat was excised with BamHI and SalI and cloned into BamHI- and SalI-digested pTRE-tight-BACGFP (Clontech). All constructs were sequence verified.

Transgenic transfection of small interfering RNA and Tet-Off expression vectors. A small interfering RNA (siRNA) pool of four siRNAs targeting the EGFR mRNA was obtained from (Dharmacon). Sequences of the sense strand were as follows: GAAGAAGACGAUAUUCAAAUU, GGAAGUAGUAGUCACGAAAUU, CCACAAAGCCAGUAAUUAUU, and GUACAAGUCGCGACGUUUU. Lum215 and HCT116 cell lines were transfected with EGFR siRNA (100 nmol/L) using the Dharmafect-4 transfection reagent (Dharmacon).

To determine the effect of mononucleotide and dinucleotide deletion mutations within the polyA repeat within the EGFR 3'-UTR, the pTRE-luciferase-EGFR 3'-UTR A13 (WT), pTRE-luciferase-EGFR 3'-UTR A12, or pTRE-luciferase-EGFR 3'-UTR A11 vector was transiently transfected into HCT116 cells in combination with the Tet-Off vector using the Profection transfection reagent (Promega). TK-Resiilla was included as a control for transfection efficiency. Twelve hours after transfection, doxycycline was added to a final concentration of 25 ng/mL, and cells were harvested 24 h later using Passive Lysis Buffer (Promega). Luciferase assays were performed using the dual luciferase assay reagents (Promega), and firefly luciferase was expressed relative to TK-Resiilla.

Xenograft assays. Colon cancer cells were grown as xenografts in severe combined immunodeficient (SCID) mice as previously described (24). For determination of sensitivity to cetuximab and panitumumab, animals were injected i.p. with PBS, cetuximab, or panitumumab (1 mg per mouse per day), for 14 d, beginning on the day of tumor cell injection. On sacrifice, tumors were extracted and volume was calculated from measurements of the smallest (s) and longest (l) diameter based on the formula volume = [(s × l) × π] / 6 and measurement of tumor weight. For siRNA-mediated EGFR knockdown in vivo, colon cancer cells were transfected with siRNA targeting EGFR or control siRNA (NT1; Dharmacon) and injected into SCID mice 24 h after transfection. Mice were sacrificed 1 wk after injection and tumor volume was quantified as above.

Immunohistochemistry. EGFR expression was determined in formalin-fixed, paraffin-embedded tissue of colon cancer cell lines grown as xenografts in SCID mice using a monoclonal anti-human EGFR antibody from Dako.

Results

EGFR is overexpressed in MSI colon cancer cell lines. EGFR expression in a panel of 28 colon cancer cell lines was determined by Western blot analysis. Remarkably, as shown in Fig. 1, we observed strong EGFR expression in several MSI colon cancer cell lines. To determine whether this high level of EGFR protein expression correlated with increased EGFR mRNA expression, EGFR mRNA expression was determined by quantitative reverse transcription-PCR (RT-PCR; Fig. 1B). Significant correlation between EGFR mRNA and protein expression was observed across the cell line panel (r = 0.65, P = 0.002, Pearson’s correlation coefficient). Immunohistochemical staining for EGFR in cell lines grown as xenografts was confirmed strong EGFR expression in several MSI colon cancer cell lines (Fig. 1C).

Overexpression of EGFR in MSI cell lines is not due to EGFR amplification. EGFR overexpression driven by gene amplification, although rare, has been reported in colon cancer (8). Although MSI tumors tend to be genomically stable, we wished to eliminate the possibility that the observed EGFR overexpression
in MSI cell lines was due to EGFR gene amplification. We therefore examined EGFR gene copy number by fluorescence in situ hybridization analysis in three EGFR-overexpressing MSI lines. As shown in Supplementary Fig. S1, no EGFR amplification was observed in the cell lines examined. Two of the three MSI cell lines showed polysomy of chromosome 7, as shown by the presence of more than two EGFR and chromosome 7 signals per nucleus. However, EGFR polysomy was also observed in three of three MSS cell lines examined and was therefore not linked to the EGFR overexpression in MSI lines (Supplementary Fig. S1).

EGFR expression does not correlate with length of the polymorphic dinucleotide (CA) repeat within intron 1 of the EGFR gene. Repetitive sequences are particularly prone to mutation in MSI tumors. Previous reports have shown that EGFR transcriptional efficiency is dependent on the length of a dinucleotide CA repeat present within intron 1 of the EGFR gene, with the efficiency of transcription proportionally reduced with increasing repeat length (10). Other studies, however, have failed to show this link (6, 25). To determine whether EGFR expression was linked to the length of the CA repeat in the colon cancer cell line panel, the length of the repeat was determined by fragment analysis. As the length of the CA repeat was heterozygous in the majority of the cell lines (Supplementary Fig. S2A), the sum of the repeat length of the two alleles was computed. As shown in Supplementary Fig. S2B, the repeat length of the two alleles ranged from 36 to 52 across the cell line panel, with a repeat length of 40 observed in the majority of cell lines. However, no significant correlation between EGFR intron 1 CA repeat length and EGFR mRNA expression was observed ($r = 0.29, P = 0.16$).

The 3'-UTR of EGFR contains a variant A13/A14 repeat sequence that is frequently mutated in EGFR-overexpressing MSI colon cancer cell lines. To determine the basis of strong EGFR expression in MSI CRC lines, the EGFR gene was examined for the presence of mononucleotide or dinucleotide repeat sequences. Although no repeat sequences were detected within the coding sequence, a 13-adenine repeat sequence within the 3'-UTR was identified (Supplementary Fig. S3A). Before determining whether this sequence was mutated in MSI colon cancer cell lines, it was important to determine whether the length of this repeat element varied among individuals in normal tissue. To determine this, we
examined the length of the EGFR 3′-UTR polyA repeat in 27 normal colon tissue samples by fragment analysis. Eleven samples (41%) contained an A14 repeat and 16 samples (59%) contained an A13 repeat at this locus. None of the normal samples showed variants at this repeat element that were fewer than 13 adenines in length.

To determine whether mutations within this repeat sequence were linked to the increased EGFR expression in MSI CRC, the length of the repeat was determined in the cell line panel by fragment analysis. As shown in Supplementary Table S1, 7 of the 11 (64%) MSI CRC cell lines analyzed showed the presence of a 1- or 2-base deletion mutation within this element, resulting in an A12 or A11 repeat. The presence of 1- or 2-base deletion mutations within the A13/A14 repeat was further confirmed by PCR amplification followed by direct sequencing in the seven mutant MSI cell lines (Fig. 2). None of the MSS cell lines tested (0 of 17) harbored a deletion mutation in the polyA tract (Fig. 2; Supplementary Table S1).

To determine the link between mutations within the 3′-UTR of EGFR and EGFR expression, we rank ordered the cell lines by EGFR mRNA expression (Supplementary Table S2). This analysis showed that four of the five cell lines that most highly expressed EGFR mRNA were MSI lines harboring a deletion mutation. In contrast, none of the five cell lines with the lowest EGFR mRNA expression were EGFR mutant. A t test revealed that cell lines harboring a deletion mutation in the EGFR 3′-UTR had significantly higher EGFR mRNA (P = 0.032) and protein (P = 0.023) expression compared with EGFR 3′-UTR WT cell lines.

Deletion mutations in the polyA(13) repeat sequence of the EGFR 3′-UTR occur at a high frequency in MSI colon tumors in vivo. To determine whether the mutation identified in the 3′-UTR of EGFR in colon cancer cell lines was also evident in freshly resected MSI colon tumors, we extended this analysis to DNA extracted from a cohort of 16 MSI tumors. Consistent with the cell line data, 11 of 16 (68.7%) MSI tumors harbored a deletion mutation (Supplementary Table S1). Of the 11 mutations identified, 5 were mononucleotide (A12) deletions (29.4%), 2 were dinucleotide (A11) deletions (11.7%), and 4 were heterozygous (A12/A11) deletions (23.5%). None of the MSS tumors tested (0 of 15) harbored a deletion mutation in the polyA tract (Supplementary Table S1).

Inactivation of Dicer in colon cancer cells has no effect on EGFR mRNA expression. As the 3′-UTR of mRNA transcripts is a major target for microRNA-mediated transcriptional and translational regulation, we next tested the hypothesis that mutation within the 3′-UTR of EGFR may lead to decreased binding of a microRNA, thus leading to increased EGFR expression. To test this hypothesis, we used the Dld-1 Dicer−/− cell line in which the helicase domain of the Dicer gene, a key processor of pre-microRNA into mature microRNA, has been deleted by homologous recombination (22). The Dld cell line was selected as it was WT for the repeat element within the EGFR 3′-UTR. However, as shown Fig. 3A, no difference in EGFR mRNA or protein expression was observed between parental and Dicer−/− Dld-1 cells, indicating that Dicer-processed miRNAs do not play a role in regulating EGFR expression.

Figure 2. Mononucleotide and dinucleotide deletion mutations within polyA repeat region in the EGFR 3′-UTR. A, representative chromatograms from WT and deletion mutant cell lines. B, validation of direct sequencing of EGFR 3′-UTR by fragment analysis. The most dominant peak represents the true fragment length. The first peak to the right of the dominant peak represents an adenine addition. Other peaks likely represent polymerase slippage.
expression. To further confirm this finding, we examined the effect of transient siRNA-mediated down-regulation of Dicer in the MSS Caco-2 colon cancer cell line. As shown in Fig. 3B, despite efficient down-regulation of Dicer protein, no change in EGFR protein or mRNA expression was observed.

**Mutation of the polyA tract within the EGFR 3′-UTR stabilizes EGFR mRNA.** An additional role of the 3′-UTR is in the regulation of mRNA stability. Initially, to determine whether mutations in the EGFR 3′-UTR resulted in increased endogenous EGFR mRNA stability, we attempted to compare the rate of EGFR degradation in EGFR 3′-UTR WT and mutant cell lines following treatment with the inhibitor of transcription, actinomycin D. However, as shown in Fig. 4A, we observed minimal decay of EGFR mRNA following actinomycin D treatment in each of the colon cancer cell lines tested. This stability was specific to EGFR, as expression of p21, which was tested in parallel, was reduced by 90% in each cell line 12 hours following actinomycin D treatment (Fig. 4B). Furthermore, this observation was unique to colon cancer cell lines, as robust decay of EGFR following actinomycin D treatment was observed in MCF7 breast cancer cells and 293T kidney epithelial cells (Fig. 4C and D).

Therefore, to directly determine whether mutations within the polyA tract of the EGFR 3′-UTR results in increased EGFR mRNA stability, we designed a Tet-Off system in which the entire 1,720-bp EGFR 3′-UTR, containing either the WT polyA(13) tract or A(12) or A(11) deletion mutations, was subcloned downstream of the luciferase gene. This construct was in turn subcloned into a pTRE-Bi-AcGFP vector in which addition of doxycycline turns off transcription of the luciferase-EGFR 3′-UTR construct (Fig. 5A). As shown in Fig. 5B, the decay in luciferase activity over time following doxycycline addition was significantly slower from constructs containing mutant polyA(12) or A(11) 3′-UTRs compared with the WT A(13) construct, indicating that truncating mutations within the A13 repeat result in a reduced rate of EGFR mRNA degradation.

**Inhibition of EGFR reduces growth of some but not all EGFR 3′-UTR mutant colon cancer cell lines.** Having identified mutations within the 3′-UTR of EGFR, which in turn was linked to increased EGFR mRNA expression, it was important to determine whether this was a driver or bystander mutation in MSI colon cancer. To address this, EGFR mRNA was down-regulated using siRNA in the LIM1215 MSI cell line, which harbors a mutation within the EGFR 3′-UTR. As shown in Fig. 6A, efficient siRNA-mediated down-regulation of EGFR was achieved. Relative to cells transfected with a nontargeting siRNA, a 40% inhibition of cell growth was observed 48 hours after EGFR siRNA transfection (Fig. 6B). Similar effects were observed in vivo where siRNA-mediated EGFR down-regulation resulted in reduced growth of LIM1215 xenografts compared with cells transfected with nontargeting siRNA (Supplementary Fig. S5). Furthermore, targeted inhibition of EGFR using the anti-EGFR antibodies panitumumab or cetuximab resulted in significant inhibition of LIM1215 cell growth in vivo and in vitro (Fig. 6C; Supplementary Fig. S4A). In contrast, however, siRNA-mediated EGFR down-regulation or pharmacologic inhibition had minimal effects on growth of a second MSI EGFR 3′-UTR mutant cell line, HCT116 (Fig. 6; Supplementary Fig. S4A). Notably, HCT116 cells harbor activating mutations in Ras and PIK3CA, two critical signaling mediators located downstream of EGFR, and which have been shown to confer resistance to EGFR inhibition (23), whereas the LIM1215 is WT at these loci.

As all the MSI lines with EGFR 3′-UTR mutations screened in our original panel harbored mutations in signaling mediators downstream of EGFR (23), or, in the case of SW48 cells, an activating G719S mutation in the kinase domain of EGFR itself (COSMIC), we screened an additional five MSI cell lines to identify further lines that harbored a mutation within the EGFR 3′-UTR but which were WT for the downstream signaling regulators K-Ras, BRAF, PIK3CA, and PTEN. A second cell line, HCA7, that contained a single A deletion mutation in the EGFR 3′-UTR but that was WT for K-Ras, BRAF, PIK3CA, and PTEN was identified. As expected, EGFR mRNA and protein levels were elevated in this line compared with four EGFR 3′-UTR WT lines (Supplementary Fig. S5).

Next, to directly test the functional significance of EGFR 3′-UTR mutations, we compared the effect of EGFR inhibition on cell growth in two EGFR 3′-UTR mutant (LIM1215 and HCA7) and the EGFR 3′-UTR WT cell line Caco-2 but which were all WT for Ras, BRAF, PIK3CA, and PTEN. As shown in Fig. 6D, cetuximab treatment resulted in greater inhibition of the percentage of cells in S phase in EGFR 3′-UTR mutant (LIM1215 and HCA7) cells compared with EGFR 3′-UTR WT Caco-2 cells. Collectively, these results indicate that mutations within the 3′-UTR of EGFR provide a growth advantage in a subset of MSI colon cancer cell lines, specifically those that do not harbor activating mutations in downstream signaling mediators.

**Discussion**

In this study, we identify and characterize a novel mutation in a polyA repeat sequence located within the 3′-UTR of the critical
signaling mediator, EGFR, in MSI colon cancers. Mononucleotide or dinucleotide deletion mutations were identified in 68% of MSI colon cancer cell lines and primary colon cancer specimens. Importantly, mutations within the EGFR 3′-UTR were associated with increased EGFR mRNA and protein expression. Notably, we also observed that this site is likely to be polymorphic, with A13 and A14 repeats observed at frequencies of 59% and 41%, respectively, in normal colonic tissue.

Figure 4. A, determination of EGFR mRNA stability in EGFR 3′-UTR WT and mutant cell lines by actinomycin D treatment. A panel of EGFR 3′-UTR mutant and WT cell lines were treated with actinomycin D for 12 h. mRNA was isolated and EGFR levels were determined by quantitative RT-PCR. B, parallel determination of p21 mRNA levels. C and D, determination of EGFR mRNA stability by actinomycin D treatment of breast (C) and kidney epithelial (D) cells.

Figure 5. Single and dinucleotide deletion mutations within the polyA repeat of the EGFR 3′-UTR increase mRNA stability. A, HCT116 cells were transiently transfected with pTRE-luciferase-EGFR 3′-UTR A13 (WT), pTRE-luciferase-EGFR 3′-UTR A12, or pTRE-luciferase-EGFR 3′-UTR A11 in combination with the Tet-Off vector and the TK-Renilla transfection efficiency control. B, 12 h after transfection, doxycycline (Dox) was added to a final concentration of 25 ng/mL. Cells were harvested at 24 h and luciferase activity was determined. Values shown are the percentage of firefly luciferase in doxycycline-treated cells relative to untreated cells. All values are corrected for TK-Renilla luciferase activity. Columns, mean of a representative experiment performed in triplicate; bars, SE. *, P < 0.05; **, P < 0.0005.
The 3′-UTR serves an important function in the posttranscriptional regulation of transcribed sequences. Mechanistically, this is mediated by altered RNA stability regulated by binding of RNABPs or, as is becoming increasingly appreciated, through binding of microRNAs. Our findings that EGFR levels were unchanged in Dld-1 Dicer−/− cells or following transient Dicer knockdown in Caco-2 cells suggest that microRNAs do not play a significant role in EGFR regulation in colon cancer cells. However, we note that whereas elimination of the helicase domain of Dicer results in the failure to process the majority of pre-microRNAs, a subset of microRNAs were found to be still processed into the mature form in these cells (22). The possibility that a microRNA within this subset may contribute to the posttranscriptional regulation of EGFR mRNA therefore cannot be eliminated.

In comparison, using a Tet-Off system, we observed that mutations in the polyA repeat of the EGFR 3′-UTR resulted in increased EGFR mRNA stability. This finding is consistent with a previous report, which showed that EGFR expression is posttranscriptionally regulated in breast cancer cells, with a 260-nucleotide cis-acting regulatory element containing four AU-rich elements within the 3′-UTR of EGFR shown to play an important regulatory role (26). Remarkably, and as shown in Supplementary Fig. S3, the polyA tract is located within the center of this element, flanked on the 5′ and 3′ ends by two ARE elements. Furthermore, our observation that EGFR mRNA is stabilized in response to actinomycin D treatment is consistent with previous studies showing actinomycin D–mediated stabilization of transcripts containing class I and II AU-rich elements (27). One possibility, therefore, is that mutations within the polyA repeat modulate the ability of AU-rich element binding proteins to interact with the EGFR 3′-UTR, thus altering mRNA stability.

The present finding that mutations in the 3′-UTR of a gene can result in increased mRNA expression is consistent with a recent report by Ruggiero and colleagues (28), who showed that a single A deletion within a polyA(8) element within the 3′-UTR of the CEACAM gene was linked to increased CEACAM expression. Furthermore, the possibility that mutations within the 3′-UTR of genes may contribute in altered gene expression in MSI colon cancers on a global scale is suggested by the finding of di Pietro and colleagues (29), who observed that a significant percentage of genes up-regulated in MSI relative to MSS colon cancer contained simple repeat sequences within their 3′-UTR.

Given the high mutation frequency observed in MSI colon tumors, particularly in repeat elements of increasing length (30), it is important to determine whether specific mutations are driver or bystander mutations. In this regard, we were able to show that siRNA-mediated knockdown of EGFR results in growth inhibition in some but not all EGFR mutant MSI lines. Notably, the EGFR 3′-UTR mutant LIM1215 and HCA7 cell lines, in which EGFR silencing induced growth inhibition, are devoid of mutations in the Ras/BRAF/MAPK and PI3K/PTEN/Akt pathways, which mediate downstream EGFR signaling. In contrast, HCT116 cells, which harbor mutations in both K-Ras and PIK3CA, were not sensitive to EGFR down-regulation or pharmacologic inhibition of the EGFR.

**Figure 6.** EGFR inhibition reduces cell proliferation in some but not all colon cancer cell lines harboring an EGFR 3′-UTR mutation. A, siRNA-mediated down-regulation of EGFR in HCT116 and LIM1215 colon cancer cells. B, siRNA-mediated down-regulation of EGFR inhibits growth of LIM1215 but not HCT116 cells in vitro. C, targeted inhibition of EGFR using cetuximab inhibits growth of LIM1215 but not HCT116 colon cancer cells in vivo. D, comparison of the magnitude of cetuximab-induced growth inhibition in EGFR 3′-UTR WT (Caco-2) and mutant (LIM1215 and HCA7) cell lines. Shown is the percentage change of cells in S phase following 24-h treatment with cetuximab (20 μg/mL). Columns, mean (n = 4 independent experiments); bars, SE.

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**Research.**
Furthermore, when response to EGFR inhibitors was examined specifically in cell lines with no known mutations in downstream signaling mediators, we observed greater growth inhibition in cell lines with mutant EGFR 3'UTR. Collectively, these findings indicate that EGFR overexpression driven by EGFR 3'UTR mutations provides a growth advantage specifically in colon cancer cells devoid of constitutively activating downstream mutations.

Mutations in the EGFR 3’-UTR and subsequent EGFR overexpression may also provide a growth advantage through other mechanisms. First, activating mutations in the EGFR kinase domain, although rare, occur in a small percentage of colon tumors (31, 32). Consistent with this finding, among the cell lines examined in the present study, SW48 cells, which we showed to have a mutation in the EGFR 3’-UTR and express high levels of EGFR, also harbor an activating G719S mutation in the EGFR kinase domain (COSMIC database, Sanger Institute), as observed in lung tumors (33). Mutations in the polyA repeat region of the EGFR kinase domain (34). Notably, in this study, the authors found that the KM12 cell line, which we found to harbor a mutation in the EGFR 3’-UTR, underwent apoptosis in response to siRNA-mediated EGFR down-regulation when cultured in low-glucose medium. This finding raises the possibility that mutations in the EGFR 3’-UTR and subsequent overexpression may contribute to cell survival in a larger subset of MSI tumors depending on the growth conditions of the tumor.

In conclusion, we have identified a novel mutation in the 3’-UTR of EGFR in a high percentage of MSI colon tumors. This mutation, through enhanced EGFR mRNA stability, results in EGFR overexpression. This in turn provides a growth advantage to a subset of MSI colon cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

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