

A dynamic interplay between alternative polyadenylation and microRNA regulation: Implications for cancer (Review)

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Abstract. Alternative polyadenylation and microRNA regulation are both mechanisms of post-transcriptional regulation of gene expression. Alternative polyadenylation often results in mRNA isoforms with the same coding sequence but different lengths of 3' UTRs, while microRNAs regulate gene expression by binding to specific mRNA 3' UTRs. In this sense, different isoforms of an mRNA may be differentially regulated by microRNAs, sometimes resulting in cellular proliferation and this mechanism is being speculated on as a potential cause for cancer development.

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1. Introduction

As is a fact that only a small fraction (~1.5%) of the genetic material in the human genome codes for protein, most genomic DNA is non-coding but involved in the regulation

of gene expression (1,2). Specifically, there are two levels of regulation for gene expression: transcriptional regulation controls whether a gene is transcribed or not and to what extent; post-transcriptional regulation controls the fate of the transcribed RNA molecules, including their stability, translation efficiency and subcellular localization (3). Regulation in each level requires several multi-component cellular machines, each carrying out a separate step in gene expression pathway. Instead of working as a simple linear assembly line, gene expression machines couple extensively to form a complex network to coordinate their activities, maximizing the efficiency and specificity of each step in gene expression (4,5). Recent years have witnessed an increased appreciation for the importance of post-transcriptional regulation in mammalian organisms. The same primary transcript can generate a number of different isoforms by processing steps such as alternative splicing or polyadenylation (6-8). New classes of non-coding RNA genes have been described, including the abundant and conserved class of microRNAs (9-13). Alternative polyadenylation often results in mRNA 3' UTRs of different lengths, while microRNAs regulate gene expression by binding to specific mRNA 3' UTRs. Earlier studies have formulated the association between alternative polyadenylation and nonsense-mediated decay (14-18), A-U-rich element mediated decay (19-21) and mRNA surveillance (22), we have good reason to deduce that there should also be interplay between alternative polyadenylation and microRNA-mediated decay. In this review, we address how alternative polyadenylation and microRNAs will tether to each other for a fine tune of gene expression and its potential link to disease development, and cancer in particular.

2. MicroRNA

MicroRNAs are evolutionarily conserved, endogenous, single-stranded, non-coding RNA molecules of ~22 nt in length that function as post-transcriptional gene regulators (23). It has been commonly maintained that microRNAs may be able to regulate ≤30% of the protein-coding genes in the human genome (24), while later study indicated that >60% of human protein-coding genes have been under selective pressure to be regulated by microRNAs (25). The mature microRNA, with the help of RNA-induced silencing complex, acts by binding to the 3' UTR of target mRNAs of protein-coding genes

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and inhibiting their translation, which has several possible consequences: it can result in the cleavage of mRNA (26) or the repression of productive translation (27) or even the destabilization and reduction in the mRNA concentration by accelerating poly(A) tail removal (28).

The current consensus is that microRNA target specificity is determined by both sequence matching and target accessibility (29). The critical region for microRNA binding is nucleotides 2-8 from the 5' end of the microRNA, called the 'seed region', which binds to its target site on a given mRNA by exact Watson-Crick complementarity. Asymmetry is the general rule for the matching between a microRNA and its target (30), in that the 5' end of the microRNA tends to have more bases complementary to the target than the 3' end does. Moreover, because it is an energy consuming process to free the base pair within mRNA in order to make the target accessible for microRNA binding, secondary structures are also required for target recognition to occur. In truth, microRNA preferentially targets 3' UTR sites that do not have complex secondary structures and are located in accessible regions of the RNA based on favorable thermodynamics (31).

3. Polyadenylation

The architecture of mammalian pre-mRNA consists of coding regions and non-coding regions. The coding regions are included within a translational start codon and a stop codon, while the non-coding regions locate separately at the 5' and 3' end of mRNA (3). The 3' end of mRNAs is generated by cleavage followed by polyadenylation (32), which is the last step of the post-transcriptional processing. Three sequence elements precisely determine the site of 3' end cleavage and polyadenylation in mammalian pre-mRNAs: the poly(A) signal (highly conserved hexanucleotide AAUAAA) or its close variants, the actual polyadenylation site (PAS) 11-30 nt downstream of the signal and the G-U-rich sequence 14-70 nt downstream of the PAS (33). Mainly five protein factors are involved in the polyadenylation process: cleavage and polyadenylation specificity factor (CPSF), cleavage stimulatory factor (CstF), poly(A) polymerase (PAP), cleavage factors I and II (CFI and CFII) and poly(A) binding protein (PABP) (34). These factors bind as a complex on the precursor RNA prior to the cleavage and polyadenylation reactions. The CPSF recognizes the AAUAAA sequence and the CstF binds to the G-U-rich sequence. The CFI and CFII cleave the RNA and generate a 3' end for polyadenylation. The PAP performs polyadenylation through two stages with the help of PABP, adding and extending poly(A) tail to the full ~200 residue length. The 3' UTR of mature mRNA transcript runs from the stop codon to the PAS, where pre-mRNAs are polyadenylated (35).

4. Alternative polyadenylation

Alternative polyadenylation is defined as use of more than one polyadenylation sites, which is prevalent in at $\geq 50\%$ of genes in mammalian genomes, producing mRNAs with different 3' UTR regions from a single transcript (36-38). At the end of the past century, Edwards-Gilbert *et al.* categorized alternative polyadenylation into three models, according to their distinct exon arrangement and PAS selection (35). A large number of

genes have multiple PASs within the 3' UTR in the terminal exon (39), arranged one behind the other in tandem and certain regulatory elements may posit between the PASs which can influence the stability or translatability of the longer mRNA. Another set of genes have an exon which is a composite of 3' and 5' splice sites followed closely by a PAS. Such composite exon can serve as either internal or terminal and encode different protein based on circumstances. The genes in the third model have two or more alternative 3' terminal exons encoding different C-termini. They are processed into different mRNAs either by using the first 3' terminal exon and its PAS or by skipping over that exon entirely and using the second 3' terminal exon with its PAS. However, later researchers combined the last two models and classified alternative polyadenylation into two categories, namely splicing-independent and splicing-dependent (40). In the first case, tandem PASs are located in the same 3' exon and the UTR will consist of a constitutive part followed by an alternative part, producing mRNAs that differ solely by the length of the 3' UTR. In the case of alternative terminal exons, the different 3' UTR will generally not share common sequence and the choice of downstream final exon is likely coupled with suppression of the 3' splice site of the more upstream one, producing mRNAs that differ by their coding sequences (Fig. 1). Although both appeared as alternative 3' UTRs, the functional consequences of these two types of alternative polyadenylation are obviously different. It is much easier to deduce that splicing dependent alternative polyadenylation encodes different protein products through the generation of different mRNAs (41,42); while splicing independent alternative polyadenylation is more likely to generate mRNA products with differential stability, which will ultimately lead to more or less protein product, depending on enhanced or diminished RNA half-life (19,43-46).

5. MicroRNA targets in alternative 3' UTRs

Splicing independent alternative polyadenylation produces transcript isoforms with 3' UTRs of different lengths, containing a constitutive segment and an alternative segment. If a microRNA target is located within the alternative part of the 3' UTR, the shorter transcript should be target free and should escape microRNA-mediated inhibition, while the longer transcript should be inhibited (47-49). Such variations are very common. It has been estimated that two thirds of targeted genes have alternative 3' UTRs, with 40% of predicted target sites located in alternative UTR segments. Based on whether the target sites fall within constitutive and/or alternative UTR segments, Majoros and Ohler classified microRNA target genes into two broad categories (50). Constitutive targets encompass predicted target genes with constitutive UTRs, as well as genes with alternative UTRs, in which all sites are located within the constitutive UTR regions. This category accounts for 56.6% of all targets. Alternatively targeted genes have at least one target site located in an alternative 3' UTR segment, which was further categorized into on/off targets and modulated targets. On/off targets are alternative targets in which all target sites fall exclusively into alternative UTR regions (accounting for 23.7% of all targets), whereas modulated targets contain alternative targets with sites in both constitutive and alternative UTR regions (accounting for 19.6% of all targets). This

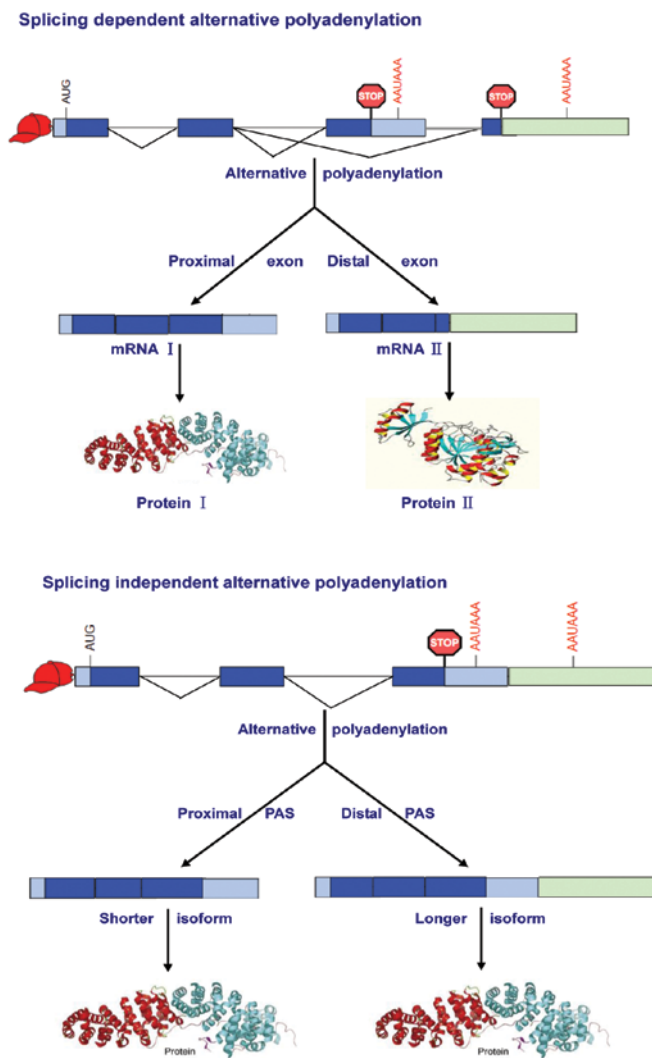


Figure 1. Splicing-dependent alternative polyadenylation and splicing-independent alternative polyadenylation.

classification of target genes perfectly illustrates the functional significance of the coupling of microRNA regulation and alternative polyadenylation. MicroRNAs regulate gene expression, while alternative polyadenylation may decide whether the expression should be regulated (on/off targets) and to what extent it should be regulated (modulated targets) (Fig. 2).

6. MicroRNA targets in COX2 gene: an example

Cyclooxygenases (COXs) are the key and rate-limiting enzymes in the production of prostaglandins (51). Two separate COX genes, *COX-1* and *COX-2*, have been identified, divergent strikingly in their regulation of expression. *COX-1* is constitutively expressed, while *COX-2* is strongly induced in response to activation by hormones, pro-inflammatory cytokines, growth factors, oncogenes, carcinogens and tumor promoters (52). The *COX-2* gene is made up of 10 exons; the 3' UTR is contained within the last exon. The *COX-2* 3' UTR is larger than average, encompassing ~2.5 kb, with several polyadenylation signals. Two of the *COX-2* polyadenylation signals are commonly used (53), the proximal one is AUUAAA (604-609 nt from

the stop codon) and the distal one is AAUAAA (2485-2490 nt from the stop codon). The proximal signal was weaker than the distal signal and the estimated ratio of proximal/distal polyadenylation signal usage was 1:3. The use of the proximal polyadenylation signal results in a ~2.6 kb mRNA, while the use of the distal polyadenylation signal results in a ~4.5 kb mRNA. These two mRNAs were exactly the same in their coding sequences, but differ in the length of their 3' UTRs, containing a 0.6-kb constitutive segment followed by a 1.9-kb alternative segment (54). Within the 1.9-kb alternative segment of *COX-2* 3' UTR, there is a microRNA binding site (seed region 1736-1743 nt from the stop codon) for both *miR-101* and *miR-199a*. *In vitro* gain- and loss-of-function experiments show that *COX-2* expression is posttranscriptionally regulated by these two microRNAs (55). Since there is no microRNA target site confirmed in the constitutive segment of the *COX-2* 3' UTR, it should currently be recognized as an on/off target. The transcription of the longer isoform of *COX-2* mRNA will turn on the target, while the transcription of the shorter isoform will turn it off.

According to 'TargetScan' prediction, there should be two microRNA target sites within the 3' UTR of *COX-2*. The distal target is exactly that mentioned above, within the alternative segment, while the proximal target is located in the constitutive segment (seed region 257-264 nt from the stop codon), predicted to be targeted by *miR-26*. However, the proximal target is not experimentally verified yet. The *COX-2* will change from an on/off target to a modulated target depending on whether the proximal target is a true target or not. If it is a true target, then the longer isoform of *COX-2* mRNA will have two target sites and be regulated by two microRNAs, *miR-26* and *miR-101/ miR-199a*, the shorter isoform will be targeted only by *miR-26*. That is, both the long and the short isoforms will be regulated, but to different extent. In this sense, *COX-2* gene should modulate the extent of its regulation through switching from one isoform to the other, or by varying ratios between the two isoforms.

7. Switch in the use of alternative PAS, a way to cellular proliferation

The longer and the shorter isoforms of mRNA have different regulation efficiencies by microRNAs, resulting in different levels of protein expression. Will there be any preferential in the use of the isoforms under certain cellular circumstances? An interesting study (56) provides evidence that a switch in the use of alternative PASs is part of a defined program for cellular proliferation. In this study, Sandberg *et al* (56) developed a quantitative method to compare the expression of the constitutive segment relative to the alternative segment of the 3' UTR for genes with tandem PASs during T cell activation. The expression pattern was found to differ markedly between resting and stimulated primary T cells. Specifically, stimulation decreased the use of the alternative segment of the 3' UTR in 86% of the genes tested. This reduction was not associated with significant changes in median mRNA levels, indicating that the effect is due to a switch in PAS usage. Since activation of hematopoietic cells is often associated with a dramatic increase in proliferation, the authors hypothesized that the decrease in the use of the longer isoform, which

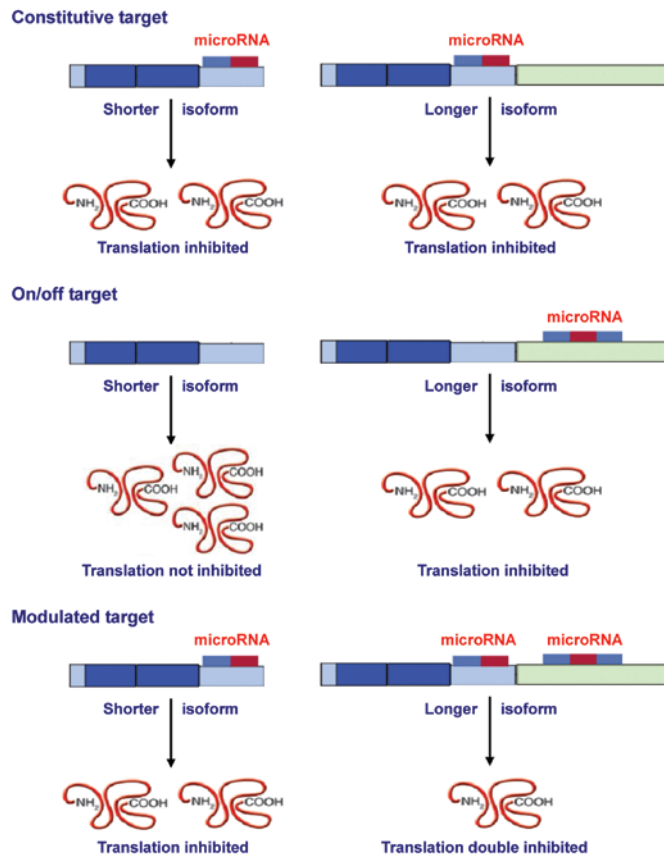


Figure 2. Three categories of microRNA targets: constitutive target, on/off target and modulated target.

leads to a shorter 3' UTR, might be associated with cellular proliferation. Interestingly, *Hip2*, one of the genes tested by Sandberg *et al*, contains in the alternative segment of its 3' UTR target sites for *miR-21* and *miR-155*, both of which are expressed in activated T cells. Overall, levels of *Hip2* mRNA were very similar between resting and stimulated T cells. However, upon T cell activation the relative level of the alternative segment of 3' UTR decreased, while protein level increased substantially. Furthermore, elimination of the target sequences for both *miR-21* and *miR-155* from *Hip2* 3' UTR increased expression level similar to that containing only the constitutive segment of 3' UTR. These results show that changes in PAS usage can control the impact of microRNA. Specifically, the use of the shorter isoform can avoid the regulation by microRNA and then enhance cellular proliferation (57).

Although it often escapes microRNA regulation, the shorter isoform of the alternatively polyadenylated mRNA is not always upregulated. In their study, Ghosh *et al* (58) found a microRNA mediated upregulation of the longer 3' UTR of the mouse cytoplasmic β -actin gene. This gene, also named *Actb*, generates two alternative transcripts terminated at tandem PAS. The longer isoform was expressed at a relatively lower level, but it conferred higher translational efficiency to the transcript. In fact, the longer isoform harbours a conserved binding site for *mmu-miR-34*. Sequence specific anti-miRNA molecule, mutations of the microRNA target

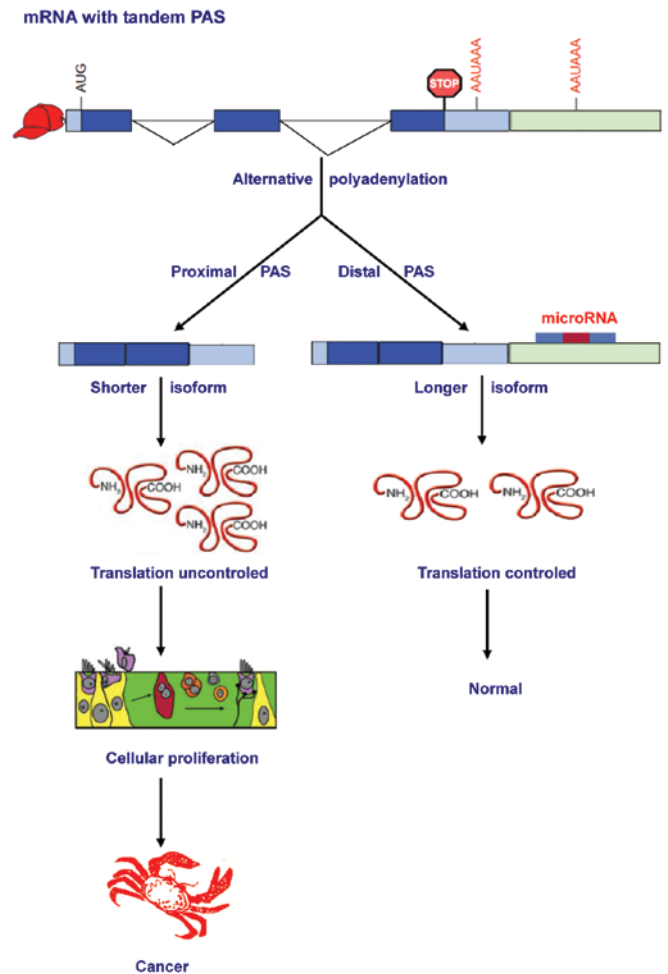


Figure 3. MicroRNAs differently regulate the longer isoform and the shorter isoform of mRNA and cause cellular proliferation and cancer.

site in the 3' UTR resulted in reduced expression and the expression could be restored by a mutant microRNA complementary to the mutated target site. These results together suggest that *miR-34* specifically target the longer isoform of β -actin transcript and upregulate the target gene expression, indicating that it is not always the shorter isoform that contributes to cellular proliferation.

8. Interplay between alternative polyadenylation and microRNA, a potential cancer link

This ability has been shown in the above-mentioned *COX2* gene in colorectal cancer cells. Instead of the 4.5-kb isoform, the 2.6-kb isoform of *COX-2* mRNA lacking the *miR-101/miR-199a* target of the 3' UTR was selectively stabilized upon cell growth to confluence, suggesting that *COX2* mRNA may escape regulation in cancer cells through alternative PAS usage (6). The general association of the use of shorter 3' UTR isoforms, avoidance of regulation by microRNAs and enhanced proliferation leads to the speculation that in some cases a shift toward expression of proximal PAS isoforms may be required to evade regulation that would otherwise restrict cell cycle progression. This is in agreement with the observation that microRNAs influence many aspects

of cellular proliferation and cell cycle progression (59,60) and accordingly downregulation of microRNA expression promotes cellular transformation and tumorigenesis, which is commonly observed in cancer (61,62) (Fig. 3). Many genes, under evolutionary selection, have the ability to enrich or deplete their microRNA binding sites through changes in the lengths of their 3' UTRs (30). 3). Earlier studies have shown that increased levels of COX2 protein were present in both human and animal colorectal tumors, whereas the normal intestinal mucosa displayed low to undetectable COX2 expression (64,65). Similarly, elevated COX2 levels resulting from defects in its regulation was also observed in many other solid malignancies, including breast, lung, prostate, pancreas, bladder, stomach, esophagus and head and neck cancers (66-73). Therefore, it is reasonable to deduce that interplay between alternative polyadenylation of *COX2* and *miR-101/miR-199a* regulation may contribute, at least in part, to the development of cancer.

A cell cycle regulating gene (74), *cyclin D1* (*CCND1*) has two major spliced transcripts, *cyclin D1a* and *cyclin D1b*. The *cyclin D1a* has a wild-type isoform of 4.5 kb in length with a full-length 3' UTR and another short isoform of 1.5 kb in length with a truncated 3' UTR (75). Containing such a long 3' UTR, the wild-type *cyclin D1a* was regulated by a number of microRNAs, as indicated in certain algorithms. A regulatory feedback loop was assumed between *cyclin D1* and microRNA regulation, in which *cyclin D1* induces the expression of certain microRNAs, in turn, these microRNAs limit the proliferative function of *cyclin D1*, thus regulating cellular tumorigenesis (76). When this feedback loop is broken, *cyclin D1* will be dysregulated and overexpressed, which is commonly seen in many types of cancer, including breast, lung, colorectal cancer and lymphomas (77-80). Many strongly proliferative tumors have exceptionally high *cyclin D1* mRNA levels and preferentially express short *cyclin D1a* mRNA isoform. This short isoform lacks many mRNA destabilization elements present in the wild-type *cyclin D1a* mRNA, including a target site for *miR-16-1* (81). Thus, truncated *cyclin D1a* mRNA deletes *miR-16-1* binding sites within the *cyclin D1* mRNA 3' UTR and alters the microRNA regulatory feedback loop, leads to increased total *cyclin D1* mRNA and increased *cyclin D1* protein expression and then contributes to cancer development.

9. Future perspectives

By truncating their 3' UTRs, certain genes can escape the regulation of microRNAs, as well as other RNA-binding proteins and thus proliferate. Just like the gecko, breaking its tail for escape in time of danger, saving itself from being injured. This pattern of tail truncation has already been implicated in the process of disease development, including cancer. A fairly large proportion of genes have different isoforms of 3' UTR and there are full of mysteries contained in the alternative segment of the 3' UTR, besides nonsense mediated decay, A-U-rich element mediated decay and mRNA surveillance, microRNA mediated decay should be an important one. Future studies are needed to investigate the association of the interplay between alternative polyadenylation and microRNA regulation in clinical settings,

which will facilitate our understanding of disease etiology and even helpful in our efforts to work out new ways of disease prevention and treatment.

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