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OncomiR Structure and Network Prediction on *Adenomatosis Polyposis Coli* (*APC*) Gene Silencing Regulation in Colorectal Cancer

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Abstract

The emergence of colorectal cancer cells is associated with the inactivation of the adenomatosis polyposis coli (APC) gene which increases the activity of ß-catenin, one of which is due to oncomiRNA (cancer-inducing microRNA). miR-135a/b-5p and miR-494-3p were thought to be involved in silencing the APC gene and increasing cell proliferation and could potentially be used as anti-miR targets. However, there is a need for an in-depth evaluation of the involvement of the oncomiR as a therapeutic target in preventing the formation of CRC. Therefore, this study aimed to predict the mechanism of inhibition of oncomiR hsa-miR-135a/b, and has-miR-494-3p against APC in the Wnt/ß-catenin signaling pathway. This research was conducted through *in silico* analysis using a web-based application to describe the stability of the secondary structure, binding position on mRNA, and conserved nucleotides that support biological activity. The data obtained were then used to develop miRNA interaction networks with APCs on the CRC-associated Wnt/ß-catenin signaling pathway. This study suggests that miR-135a-5p, and miR-135b-5p probably evolved earlier in the evolutionary evolution of the conserved oncomiR CRC in various vertebrate species, whereas miR-494-3p is more conserved and commonly found in mammals. The biological activity of miR-494-3p is likely to be more stable and patent to bind to APC gene mRNA and trigger CRC cell proliferation. Furthermore, miR-135a/b-5p and miR-494-3p have the potential to be developed as targets for anti-miR-based transcriptomic therapy as well as for early diagnosis of CRC development. Anti-miR therapy will likely need to involve more than 1 miRNA, as each gene has more than 1 miRNA binding site.

Keywords: ß-catenin, Cancer cell proliferation, miR-135a/b, miR-494, Wnt

Introduction

Colorectal cancer (CRC) is one of the 4 most common types of cancer in the world [1]. In 2020, CRC sufferers reached more than 1.14 million people with a mortality rate of 5.8 million, and is predicted to increase to 13.8 million by 2030 [2]. The development of CFCs is often associated with a decrease in the activity of the adenomatous polyposis coli (APC) gene either due to mutations [3,4], as well as the silencing mechanism by oncomiR (a miRNA sequence that plays a role in increasing the risk of cancer) [5,6]. APC is classified as a tumour suppressor related to the stimulation of apoptotic activity by suppressing the action of ß-catenin (oncogene). In addition, APC proteins have a variety of vital functions, including cell cycle control, cell attachment in other tissues, cell morphology and polarization to cell movement out of tissues [7]. This protein also helps ensure that the number of chromosomes in a cell produced by cell division is correct. Therefore, suppression of APC has an impact on increasing the function of ß-catenin in increasing cell survival and preventing intestinal epithelial cell apoptosis, thereby increasing the risk of cancer [8].

Recent studies have shown that decreased APC protein activity, associated with gene silencing mechanisms, is regulated by various types of specific oncogenic miRNAs that have the potential to be used as biomarkers [9], targeted therapy, and prediction of chemoresistance in CRC [6]. Several types of oncogenic miRNAs affect APCs thereby upregulating the Wnt/ß-catenin cascade, including hsa-miR-135a/b-5p, and miR-494-3p [6]. The oncomiR undergoes significant ur-regulated expression in the early stages of CRC malignancy by triggering a decrease in the rate of apoptosis and increasing the proliferation of cancer cells. Thus, the presence of oncomiR in blood serum has the potential to be used as a marker of early stage malignation of CRC in the body [10,11]. Through miRNA, the development of CRC can be known with certainty so that it can be used as a reference in the development of both prevention and treatment measures [9,12].

However, further evaluation is needed regarding the involvement of these miRNAs as therapeutic targets in preventing the formation of CRC [13]. Thus, an in-depth understanding of the molecular mechanisms and pathways that underlie the contribution of oncogenic miRNAs is important as an initial step for diagnosis and targeted therapy in the development of CRC. Therefore, this study aimed to predict the mechanism of inhibition of oncomiR hsa miR-135a/b, and has-miR-494-3p against APC in the Wnt/ß-catenin signalling pathway. This study discusses the molecular mechanism of oncomiR as the 1st step in the development of early diagnostic and targeted therapy for the treatment of CRC.

Materials and methods

This study used 3 oncomirs thought to be involved in the regulation of gene expression through gaining the function of Wnt/ß-catenin signalling pathway. Three miRNA-influenced cancer developmental pathways, including has-miR-135a-5p, has-miR-135b-5p, and hsa-miR-494-3p, were identified and mapped from the Kyoto Encyclopaedia of Genes and Genomes website (https://www.genome.jp/kegg/pathway.html). The KEGG information obtained is then arranged in a cancer development flow chat as shown in **Figure 1**.

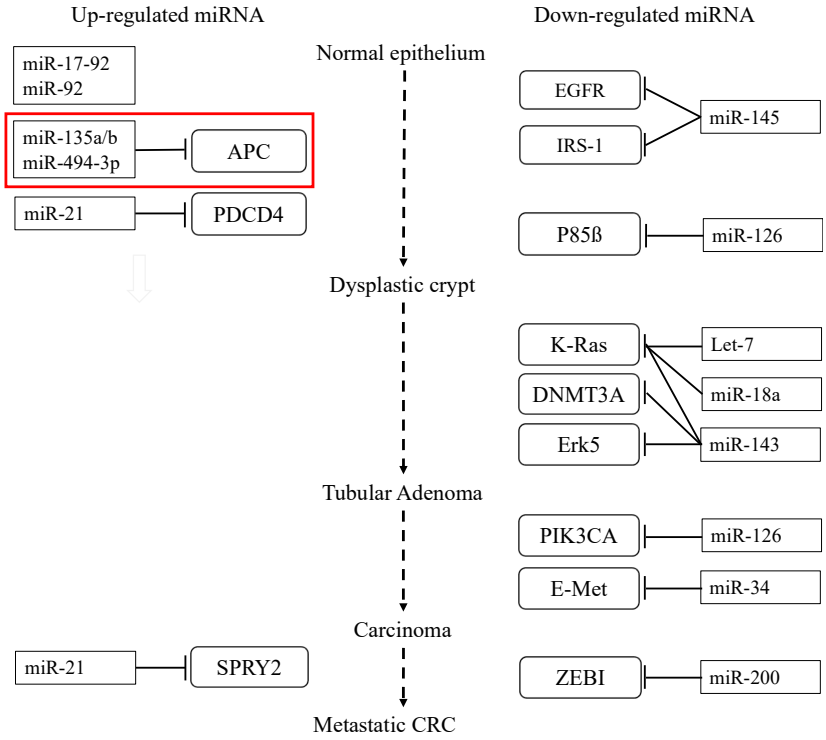

Figure 1 Regulated miRNA and affected gene during CRC development. Targeted pathway is showed by the red-line rectangle.

Insilco analysis of the mechanism gene silencing of targeted miRNA against APC genes was built based on registered miRNA sequences and modelling of the web-based programs used. The 3 miRNA data in this study were collected, analysed and elaborated to show specific characteristics, including the size and position of miRNA alignment in the 3'-untranslated region (3'-UTR) APC mRNA, nucleotide sequence, thermodynamic stability, hairpin structure, loop structure length and interactions with target genes following previous studies [14].

The current study used a miRNA database extracted from miRbase (https://www.mirbase.org/index.shtml) [15], which includes premature and mature-miRNA sequence data, annotation information, and targeted genes. The obtained premature sequences were then converted into hairpin structures to investigate the 2 loop and rod structures using RNAfold WebsServer (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi) [16]. The information obtained was used to study rod-loop structure and

stability, predict single-stranded RNA secondary structure, partition function, and minimum free energy (MFE) structure.

Targeting of the APC gene was carried out using the web program TargerScanHuman (https://www.targetscan.org/vert_80/) with a total targeted mRNA length of 3827 bp. Results were read based on the conserved portion of the APC mRNA recognized by the target miRNA. Identification of the seed type in the 3'-UTR APC mRNA was carried out based on the canonical miRNA attachment site as shown in **Figure 2**.

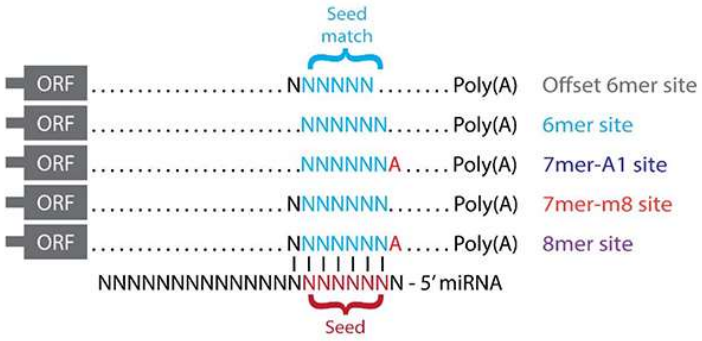

Figure 2 Canonical site type explanation (source: https://www.targetscan.org/docs/7mer.html).

The identification of the binding site was also matched with the presence of mature-miRNA sequences previously obtained from miRBase and TargerScanHuman. The compiled data were then used as a justification for the validity of the miRNA binding formation.

Results and discussion

Results

The secondary structure of miRNA is formed based on the hairpin precursor showing positional entropy on the nitrogenous bases that make up the miRNA. Based on the results of the analysis, the lowest range of entropy values was obtained from the miR-135a-5p structure, while the highest was from miR-494-3p (**Figure 3**). An understanding of entropy indicates the position of the conserved base in mature miRNAs and ensures that the nitrogenous base position found is biologically rational.

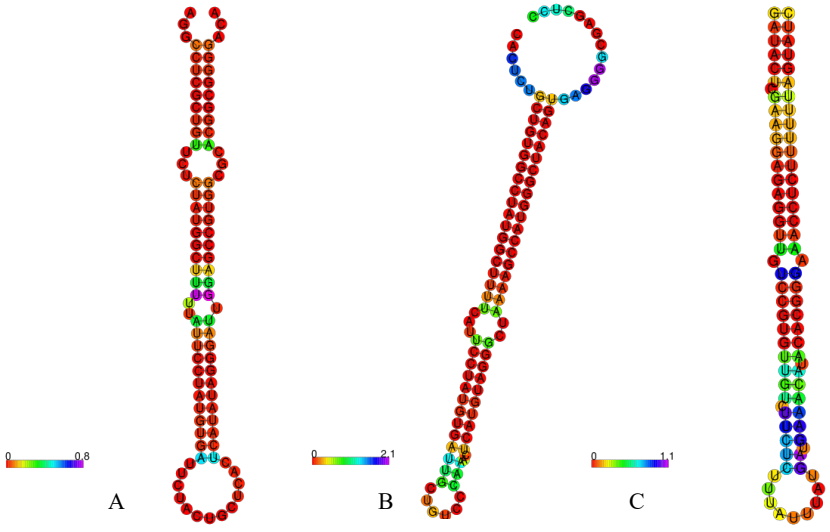

Figure 3 Hairpin structure of premature miR-135a-5p (A); miR-135b-5p (B); and miR-494-3p (C).

The results of secondary structure analysis of miRNA precursors showed that the position of the limiting nucleotide at the end of the loop with stem had a higher entropy value, indicating that the level of stability of the nucleotide was probably more converted than its neighbour. Based on the secondary structure simulation also shows that miR-135a-5p has more loop structures than miR-135b-5p. Furthermore, more conserved nucleotide positions were found in miR-494-3p.

Secondary structure formation in miR-135a/b and miR-494 requires a minimum energy consumption ranging from 33.90 to 46.70 kcal/mol in dot-bracket notation. Meanwhile, in the hairpin series, the minimum energy requirement ranges from 34.74 to 47.95 kcal/mol (**Table 1**). This represents the binding free energy of the primary structure (single strand) which is represented in the energy in dot-bracket notation into a microRNA duplex structure in the form of a hairpin (thermodynamic ensemble) [17].

Table 1 Result for minimum free energy prediction in hairpin structure of targeted miRNA.

miRNA	Minimum free energy (ΔG)* (kcal/mol)		f (%)	Ensemble diversity
	In dot-bracket notation	Thermodynamic ensemble		
miR-135a-5p	–42.60	–42.89	62.26	1.18
miR-135b-5p	–46.70	–47.95	13.22	8.48
miR-494-3p	–33.90	–34.74	25.43	4.25

Note: * The centroid secondary structure with a minimum free energy; ΔG = The free energy of the thermodynamic ensemble; f = the frequency of the MFE structure in the ensemble. RNA parameters calculation score is described in Mathews *et al*. [18].

The ensemble frequency represents the possible stability of a single structure under Boltzmann Weighted conditions (the probability that a system in thermal equilibrium occupies a state position). The higher the percentage value of the frequency ensemble indicates the stability of the secondary structure due to the high interaction between complementary nucleotides. Meanwhile, ensemble diversity shows the average base pair distance between the stem and loop structures in the coupled structure. The greater the ensemble diversity value indicates that each nucleotide forms a complementary and interrelated structure.

The identification of mature sequence miRNAs showed the number of 23 nucleotides located at the 16/17th nucleotide sequence in miR-135a/b. Although targeting mRNA from the same gene, the mature sequence of miR-494 has a different sequence (**Table 2**). This shows that the silencing mechanism regulated by miRNA has various binding regions with various miRNA regulators.

Table 2 Prediction of mature miRNA based on nucleotide position and sequence.

miRNAs	Mature sequence	Start-end base pair position
miR-135a-5p	5'–UAUGGCUUUUUAUUCCUAUGUGA–3'	17/39
miR-135b-5p	5'–UAUGGCUUUUCAUUCCUAUGUGA–3'	16/38
miR-494-3p	5'–AGGUUGUCCGUGUUGUCUUCUCU–3'	15/37

Meanwhile, based on the identification of the target gene, the binding position of miR-135a/b-3p was matched, which is a conserved part of the majority of vertebrates, while miR-494-3p is probably conserved in the majority of mammals with a fixed seed type of 7mer-A1 (**Figure 4**). This suggests that the miR-135a/b sequence may develop earlier than miR-494 and that additional regulation appears to regulate post-transcriptional APC expression in CRC mutations.

The positioning model of seed type on miR-135a/b is on the same mRNA nucleotide sequence and is of 8mer type. Meanwhile, miR-494 forms a seed match site on nucleotides at positions 62 - 68 with a seed match type of 7mer-1A. Seed match in the form of 8mer has a stronger bond than 7mer-1A formed between miR-494 and 3'-UTR APC mRNA. Furthermore, the context++ score (CS) was calculated based on 14 features as described by Agarwal *et al*. [19] showed the binding efficacy of miR-494 may be more patent than miR-135a/b (**Table 3**).

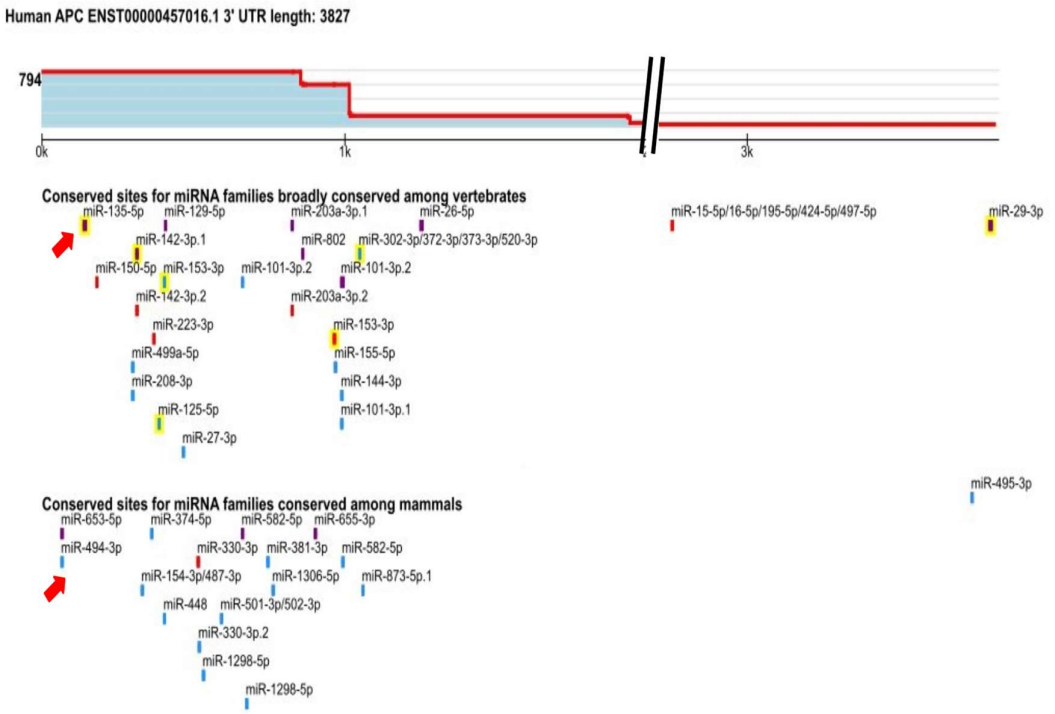

Figure 4 untranslated region of mRNA of APC is conserved sites for miRNA binding target in mostly vertebrate and mammals.

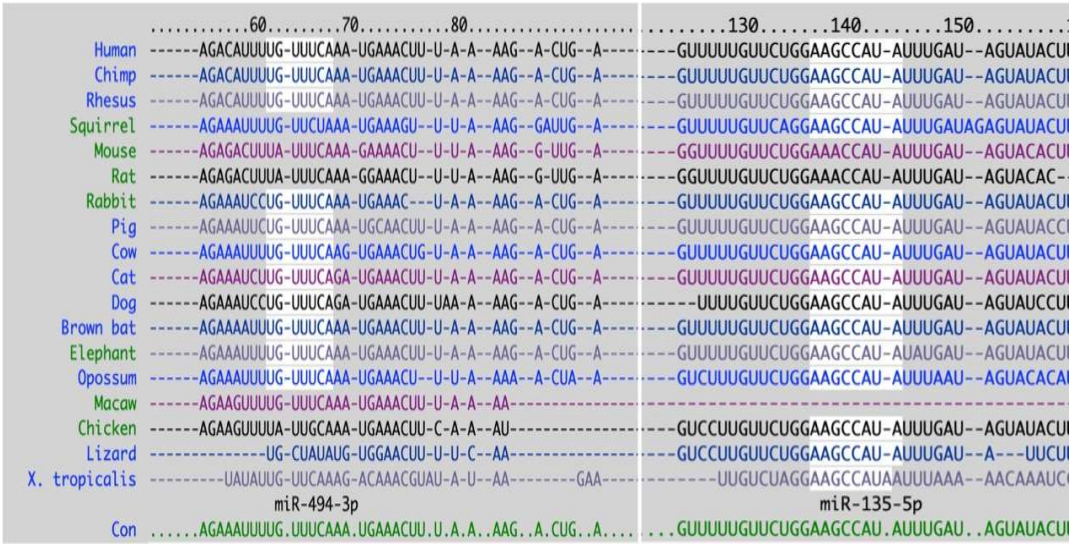

Figure 5 Seed match position of miR-135a/b-5p and miR-494-3p toward mRNA of APC gene in various vertebrate species. Sequence fragments are indicated by white lines between the nucleotide sequences, while the seed match sites are indicated by white blocks in the sequence. Dash-line shows nucleotide sequence gaps in APC gene mRNA after alignment process.

Table 3 Information for predicted consequential pairing of APC region and miRNA.

miRNA	hsa-miR-135a-5p	hsa-miR-135b-5p	hsa-miR-494-3p
Position in UTR	138 - 145	138 - 145	62 - 68
seed match	8mer	8mer	7mer-1A
context++ score	–0.25	–0.27	–0.03
context++ Percentile	93	94	62
Weighted context++ score	–0.25	–0.27	–0.03
Conserved branch	8.815	8.815	4.869
P_{ct}	0.95	0.95	N/A
Predicted relative KD	–2.927	–2.927	–3.14

Note: Context++ score and features that contribute to the context++ score are evaluated as in Agarwal *et al*. [19]; Conserved branch lengths and P_{CT} are evaluated as in Friedman *et al*. [20] with an expanded 84-species alignment as described in Agarwal *et al.* [19]. Predicted relative K_D is evaluated as in McGeary *et al*. [21].

The miRNA 135 group has variations in miR-135a and miR-135b types based on mature sequence fragments, but has the same binding site. Therefore, a representative miRNA between the 2 miRNAs was selected based on the lowest context++ value which represents the efficiency of miRNA binding to the target mRNA gene [22]. Accordingly, miR-135a was used as a representation of miR-135 although all miRNA members of the miRNA family were also predicted to target genes with the same binding site.

The results of visualization of the target miRNA ensemble diversity, showed a conserved area along 8 nucleotides in the 3'UTR of APC mRNA from various mammals (**Figure 5**). 3′-UTR contains binding sites for regulatory proteins as well as microRNAs (miRNAs). By binding to specific areas in the 3′-UTR, miRNAs can decrease gene expression of various mRNAs by inhibiting translation or directly causing degradation (**Table 4**).

Table 4 Seed position and silencing effect information of miRNA in APC region.

Targeted gene and miRNA	Seed position toward APC mRNA	Silencing Effect
mRNA-APC	5'–AAGCCAUA–3'	
hsa-miR-135a-5p	3'–UUCGGUAU–5'	Increase CRC cell proliferation through ß-catenin moderation.
hsa-miR-135b-5p	3'–UUCGGUAU–5'	
mRNA-APC	5'–UGUUUCAA–3'	
hsa-miR-494-3p	3'–ACAAAGU –5'	Increase proliferation, anti-apoptosis and triggers tumorigenesis

The oncomiR expression of miR-135a/b-3p and miR-494 may be increased through a mechanism that is not clearly understood in this study. Further research needs to map the triggering factors that increase the expression of the 2 oncomiRs, thereby suppressing the expression of the APC gene through mRNA degradation.

Discussion

In this study, it was shown that the investigated oncomiR has high potential in suppressing the activity of APC synthesis. The entropy study also showed the potential for miR-135a/b and miR-494 precursors to be secondary stable so as to produce mature miRNAs. The entropy value of miR-494 indicates that the nucleotide position with greater entropy is considered significant in compiling the secondary hairpin structure and becomes a conserved region in the miRNA family [23]. In addition, the important position of

nucleotides may have more significant functions in gene regulation and biological evolution. Thus, identification of the important position of miRNA helps in understanding evolutionary processes and predicting the function of unknown miRNAs [24]. Interesting findings in this study are that the miRNA structure is composed mostly of U nucleotides than other nucleotides, the presence of nucleotide structures that are suppressed to variations in the number of loops and the middle position of miRNAs related to the biological role of miRNAs, especially the process of maturation and gene silencing [25].

The APC protein is produced by the APC gene on chromosome (chr) 5 which acts as a tumour suppressor. APCs are involved in Wnt signalling as negative regulators of cell cycle-associated ß-catenin protein. In addition, APCs also play a role in hepatocyte growth factor (HGF)-induced cell migration [26]. APCs are also required for up-regulation of matrix metalloproteinases (MMPs) via the Janus Kinase (JNK) signalling pathway in CRC cells [4]. Functionally, APC protein acts as a stopper for ß-catenin activity through synergistic activity with Axin-1 and glycogen synthase kinase 3-α/β (GSK-3α/β) through the formation of protein complexes with repeated SAMP [27]. The protein complex then binds to ß-catenin in the cytoplasm and with the help of casein kinase 1 (CK1), results in ubiquitination and initial phosphorylation of ß-catenin which is continued by GSK-3β. Repeated phosphorylation causes N-terminal Ser and Thr residues to ubiquitinate and promote degradation by the cellular proteasome [28]. This prevents -catenin from being formed and prevents its translocation into the nucleus, where it acts as a transcription factor for proliferative genes.

The ability of APC to bind to ß-catenin which triggers degradation is a cell control mechanism against the potential for continuous cell proliferation. Thus, damage to the APC gene caused by mutations or the mRNA silencing mechanism by miRNA has an impact on increasing ß-catenin activity [29]. APC mutations in CRC cases are often associated with loss of ß-catenin and SAMP binding sites or direct interaction with the Actin protein (**Figure 6**).

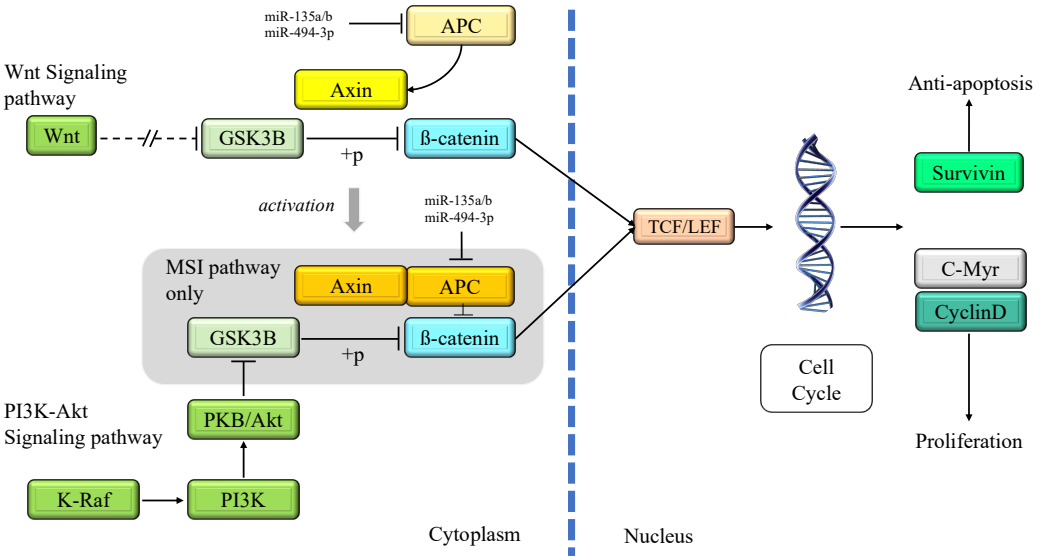

Figure 6 Inhibition target of miR-135a/b-5p and miR-494-3p against APC in Wnt signalling pathway.

In its regulation, APC requires Axin-1 and GSK-3α/β in the regulation of the Wnt signalling pathway. The attachment of the transduction signal to the Wnt protein surface receptor causes phosphorylation of Low-density lipoprotein receptor-related protein 5 (LRP5/6) so that it binds to the APC-Axin1-GSK-3α/β complex which causes inactivation so that ß-catenin is not degraded and can continue the signalling pathway to the nucleus [27,30]. Furthermore, recent studies have also shown that APC inactivation in CRC cases is also correlated with an increase in several types of oncomiR including miR-135a/b-3p [31] and miR-494-3p [32]. This is also supported in this study where miR-494 tends to inhibit the translation of APC gene mRNA more strongly than miR-135a/b-5p.

Conclusions

The APC gene has been shown to play an important role in suppressing the Wnt/ß-catenin signalling pathway associated with cell proliferation. The increase in ß-catenin activity due to the inactivation of the APC gene causes uncontrolled cell proliferation which leads to the formation of CRC. In some cases, CRC formation was also caused by silencing activity by oncomiRs, such as miR-135a-5p, miR-135b-5p and miR-494-3p which inhibited APC gene mRNA translation. This study suggests that miR-135a-5p, and miR-135b-5p likely evolved earlier in the evolution of conserved CRC development in various vertebrate species, whereas miR-494-3p is more conserved and common in mammals. However, based on the biological activity miR-494-3p is likely to be more stable and patent to bind to the mRNA of the APC gene.

Based on the biological characteristics that have been explored in this study through *in silico* analysis, it is shown that miR-135a/b-5p and miR-494-3p have the potential to be developed as targets for transcriptomic anti-miR-based therapy as well as for early diagnosis of CRC development. Anti-miR therapy will likely need to involve more than 1 miRNA, as each gene has more than 1 miRNA binding site. However, targeting oncomiR needs to be developed based on a holistic understanding of the causes of increased proliferative activity of CRC cells. This is because anti-oncomiR therapy may be less effective on CRC cells caused by mutations in the APC gene.

Acknowledgments

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