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The Promising Role of Let-7 Microrna in Colorectal Cancer: Practical Points for Clinicians

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Abstract

Colorectal cancer, one of the most common cancers, displays disproportionally high mortality, taking into consideration the enormous amount of data collected over the recent decades and the broad use of preventive colonoscopy worldwide. MicroRNAs, small, non coding RNA molecules, regulate many critical steps of the entire stages of colorectal tumorigenesis process and shed light to the in depth comprehension of the complex genetic environment that governs the process. Let-7 is the largest microRNA family studied and consists of ten mature members, which exhibit redundancy in colorectum. Its main role is to promote differentiation and depress stemness, both in normal and neoplastic colon. It represses or abrogates translation of other genes, by complementary binding to their mRNAs. It is mostly considered a tumor-suppressor, as it targets mainly oncogenes; among them K-ras is the dominant. Let-7 establishes feedback loops with the majority of its targets. Its expression levels increase over fetus development, are higher on the top *vs* the bottom of the colonic crypt and are downregulated, as normal colorectal epithelium progress to neoplasia. Although let-7's tumor-suppressive effect is dependent on the primitive or advanced stage of colorectal neoplasia, colorectal cancer tissues' let-7 levels are proportionate to cancer stage. High let-7 levels may prove a favorable prognostic and predictive biomarker. In case scientists overcome multiple limitations, the administration of mature let-7 inside colorectal cancer tissues (directly, delivering synthetic let-7 or indirectly, using a viral vector), may improve future management of CRC, since underexpressed let-7 levels favor every single stage of colonic oncogenic transformation.

Keywords: Let-7; K-ras mutation; LCS6; Biomarker; Colorectal cancer

Abbreviations

APC: Adenomatous Polyposis Coli protein

CDKs: Cyclin-dependent kinases

CRC: Colorectal Cancer

DDR: DNA damage response

DSBs: DNA double-stand breaks

EMT: Epithelial-Mesenchymal Transition

GSK-3b: glycogen synthase kinase 3b

FFPE: formalin-fixed, paraffin-embedded

HMGA2: High Motility Group A2

IGF2BP1: Igf2 mRNA binding protein 1

Let-7: lethal-7 microRNA

LCSs: let-7 complementary sites

MAPK: the pathway of MAP kinases

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miRNA: MicroRNA

NFκB: Nuclear factor of κ light polypeptide gene enhancer in B-cells

PDH-K: Pyruvate dehydrogenase kinase

PDK1: Phosho-inositide-dependent kinase 1

PI3K: Phosphatidylinositole 3-kinase

RAS-GDP: the inactive form of oncogene RAS

RAS-GTP: the active form of oncogene RAS

RISC: RNA-induced silencing complex

SNP: Single Nucleotide Polymorphism

TGF-β: transforming growth factor-β

TGF-β Rec: cell membrane receptor of TGF-β

UTR: UnTranslated Region, Wnt: Wnt signaling pathway

Introduction

Colorectal cancer is the third leading cause of cancer-related deaths on the USA [1]. Colorectal carcinogenesis is governed by the interaction between the inherited genome, the somatic genetic alterations and environmental factors [2]. MicroRNAs consist of 18-25 nucleotides and are crucial epigenetic regulators of the entirety of steps of colorectal carcinogenesis process [3-6]. They don't translate into proteins; instead, they downregulate the translation process of their mRNA-target [7]. Let-7 family, the best miRNA family studied [8], consists of ten mature members [9]. Let-7 forms RISC [7] and guides it to the 3′ UTR of its mRNA-target: partial complementarity represses mRNA translation, whereas high complementarity degrades mRNA [10].

The Increasing Number of mRNA Targets of Let-7

Mature let-7 is broadly viewed a tumor-suppressor gene, since the majority of its targets are strong oncoproteins [11-13]. It downregulates a plethora of mRNAs, mainly participating in the two major initiating oncogenic pathways in CRC (MAPK and Wnt). Furthermore, it targets other oncogenes, as c-myc [8,13], HMGA2 [14] and LIN28 A/B [8-13]). Lin28, a RNA-binding protein, promotes stemness [12,15] and inhibits differentiation [16,17]. When Lin28 is downregulated, Wnt signaling is hypoactivated and β-catenin levels are lowered [8-13,15-17]. β-catenin links Wnt and MAPK pathways, both directly, achieving mutual enhancement with RAS-GTP state and indirectly, promoting GSK-3b [18]. Therefore, let-7 downregulates MAPK pathway in a dual manner, by destabilizing RAS into RAS-GDP state and by lowering levels of β-catenin cytoplasmic pool (Figure 1).

The dominant target of let-7 is K-ras, one of the earliest mutations in colorectal carcinogenesis process [18]. Let-7 represses K-ras protooncogene [19-30] and K-ras oncogene [23-30] through ten different LCSs, located in the 3′ UTR of K-ras mRNA [31]. Let-7 silences K-ras mRNA only in case LC6 is intact. LCS1 and, mostly LCS6, are well-studied in CRC. A SNP variant in LCS6, a germ-line functional variation of T to G substitution, namely G-allele [20-31], is found in 6% of the population worldwide [25] and diminishes the binding affinity of let-7 with K-ras [30].

In wild-type K-*ras* CRCs, G-allele carriers (TG or GG) have lower levels of let-7. Therefore, G-allele reinforces the tumorigenic effect of low let-7 levels and is currently viewed as an inheritable tumor-promoting alteration [20]. A clonal selection inside the growing neoplasm favoring more aggressive clones, which bear both the hostle germline variant and the oncogene-promoting low let-7 levels is presumed [23,25], probably accomplished through the use-it-or-lose-it mechanism [20]. The ultimate result is the gradual increase of the G-allele throughout the successive stages of colorectal carcinogenesis [25]. Despite the striking differences among research works [21-30,32], the collective data imply that the interplay among three independent factors (*1* K-ras status *2* let-7's levels and *3* LCS6's genotype) determines the course and prognosis of adenomatous colorectal neoplasias.

The arsenal of let-7 targets is completed by a plethora of oncogenes. Let-7 downregulates PI3K/AKT pathway, targeting protein AKT, mTOR and PDK1[8]. It prevents dissemination of CRC cells by inhibiting IGF2BP1 [16], IFG1R and PDH-K [8]. It depresses the progress of cell cycle, as it targets cyclins (A,D1,D2,D3) and CDKs (2,4,6,25A,34A) [8,13,33]. It inhibits the antiapoptotic protein BCL-XL, enhancing apoptosis [8]. Last, it represses IL-6 and STAT-3, which are indispensable for the transition of inflammation to CRC [8,34].

Nevertheless, let-7 is capable to exhibit oncogenic properties as well. High levels of let-7 limit apoptosis, by inhibiting the death receptor Fas [35] and promote proliferation, by downregulating the antiproliferative TGF-β [10]. Let-7 suppresses innate immune reactions against CRC by inhibition of Toll-like receptor 4 [36], by inhibition of NFκB pathway [8] and by targeting mTOR RNA [37]. Last, let-7 may repress the translation of *TP53* gene [38].

The interplay between let-7 and its effectors is complex; nearly all let-7 targets behave as its reciprocal regulators, negatively in their majority. Ras negatively regulates let-7 by activation of NFκB [34] and by upregulating LIN28 *via* MAPK activated c-myc expression [8]. NFκB fosters let-7a expression by inducing its promoter [39]. Let-7 establishes a negative feedback loop with LIN28A/B [8,13,17], with HMGA2 [8,14] and with c-myc [8,35,40,41]. Wnt pathway activation increases β-catenin levels, which hyperactivates LIN28 [17]; both repress let-7. p53 protein suppresses let-7: in early stages of tumorigenesis wtp53 inhibits let-7 (directly, by binding to its promoter [42,43]) and indirectly, by inhibiting Fas [43]), whereas in late stages of CRC, mutated p53 suppresses let-7 by inhibiting its maturation process [44]). Last, but not least, let-7 targets itself; it is positively autoregulated, (mature let-7 enhances its own biosynthesis [45]) and is negatively autoregulated (it drives its own degradation in case it does not fulfill its pursuit, through the use-it-or-lose-it mechanism) [20] (Figure 1).

The Evolution of Let-7's Colorectal Levels from Early Fetal Life to Late Carcinogenesis

Mature let-7 is undetectable in early fetal life, whereas let-7's expression increases during late embryogenesis [11]. In embryonic and in adult life, let-7's major role is to promote differentiation: it is undetectable in embryonic [12] and in normal colon stem cells [11,13,17], whereas higher levels are maintained in embryonic and adult differentiated cells [11,12] and tissues [13].

Let-7 levels display striking inhomogeneity both in normal and neoplastic colon; its global mission is to establish obstacle against

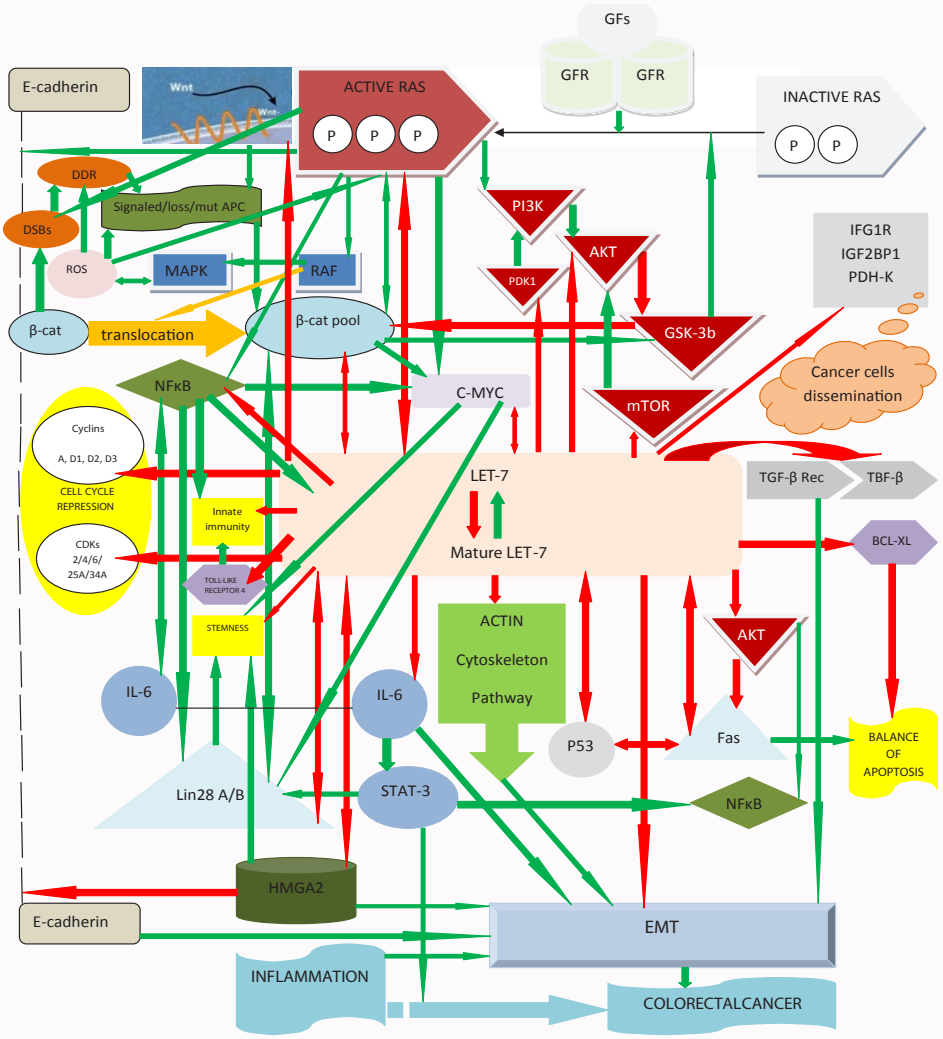

Figure I: The tumor-suppressive role of let-7 in colorectal cancer: let-7 holds a central regulatory position in the complicated cross-talk between the major pathways of colorectal tumorigenesis process; it is interdependent on multiple effectors and inhibits cell cycle, innate immunity against cancer cells, epithelial-mesenchymal transition, dissemination of transformed cells and stemness. The arrows filled with red colour indicate inhibition of the target they direct; the arrows filled with green colour indicate promotion of the target they direct (based on references 8, 10, 13, 14, 16-30, 32, 34-40, 42-45, 58-60, 65 and 87-94).

stemness. Being upregulated in stem cells, it forces and governs their differentiation [11], it controls the timing of differentiation and is upregulated upon differentiation [46]. Acting upon differentiated cells, it exerts a dual effect: *first*, it prevents their dedifferentiation to stem cells [46] and, *second*, it represses cell cycle progression and therefore halts their proliferation as they migrate towards the top of the crypt, thus preventing their neoplastic transformation [47]. Let-7 keeps differentiated cells differentiated; when this function fails, neoplastic conformation initiates [10]. As CRC is a disease of stem cells [48,49], the tumor-suppressive role of let-7 is explained by its action upon cells of the top and of the bottom of the crypt. A gradual increase of let-7 levels is thereby evident across normal crypt axis [48]: normal colorectal stem cells in the basis of the crypt express low or undetectable let-7 levels [11,13,17,47], in contrast to increasingly high let-7 content in the differentiated cells in the transition and the villous domain of the crypt [11]. Let-7 is the most representative marker of epithelial differentiation in colon [47,50].

Colonic neoplastic transformation, a process similar to reversed embryogenesis [11], presupposes loss of let-7 even from its very early stages [50]. Indeed, mature let-7 levels are underexpressed in colorectal neoplastic tissues compared to normal adjacent tissues both in premature stage (benign adenoma) and late stage (carcinoma) of the process (Figure 2) [44].

Similarly, upregulation of let-7 guides colorectal cancer stem cell transition to differentiated cancer cells [11]. Stage III/IV CRC tissues bear higher levels of let-7-a/let-7-b compared to their corresponding stage I/II [36]. Mature let-7-a levels parallels the progression of colorectal cancer [51] and let-7 exhibits increased levels in advanced colorectal cancer tissues [52-54]. In case metastases occur, let-7-a continues to increase its expression [51]. The gradual increase of the tumor-suppressor let-7 through the successive stages of CRC may reflect either the pressure of natural selection, or the under-defined role of tumor-promoting properties of let-7. Last, inflammatory stroma surrounding cancerous cells harbors up to 4 times higher let-7 levels compared to their paired cancer cells [36], implying that let-7 may regulate the stroma/cancer cells interaction inside the growing colorectal neoplasia (Figure 2).

The Decalogue of let-7's Action in Colorectum

1. Mature let-7 members, though don't harbor absolutely the

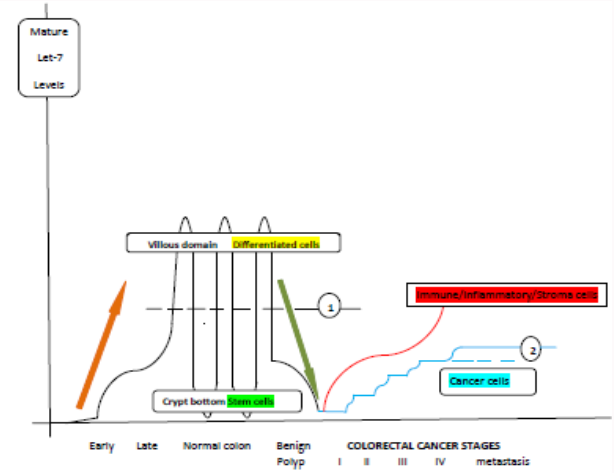

Figure II: The suggested relative concentrations of mature let-7 in colorectal cells over the phases of colon evolution: fetal colon, normal adult colon, neoplastic adult colon. Let-7 levels in colon mucosa increase gradually during development of fetus and reach approximately the average levels of the adult colon mucosa (line 1) after birth. A gradient is established from the bottom of the crypt (low or undetectable let-7 levels) to the villous domain on the top of the crypt (high let-7 levels) across the adult colon crypt axis. Colorectal adenoma cells bear intermediate let-7 levels *i.e.* levels between these of normal colorectal mucosa (level 1) and those found on the first stage of colorectal cancer. Colorectal cancer stages display incremental levels of let-7 from stage I to stage IV, and probably from stage IV to metastatic disease (blue curved line). Nevertheless, the maximum levels of let-7 in colorectal cancer cells (line 2) are maintained below the average normal colorectal mucosal levels (line 1). Stroma cells surrounding cancer contain increased let-7 levels (red curved line), compared to their paired cancerous colorectal cancer cells. According to the progress of let-7 levels, colorectal carcinogenesis process (green arrow) is indeed the inversion of the embryogenesis process (brown arrow) (based on references 12, 13, 17, 36, 43, 45-55, 61, 69, 71 and 76).

same sequence, they greatly resemble one another [45]. They share identical seed sequence, crucial for target recognition [55], which ensures that their actions overlap, both in normal and neoplastic colon [56].

2. Let-7 prevents all the hallmarks of cancer [57]. Low let-7 levels force growing cells to sustain proliferation signaling and to evade tumor suppressors [8,10], enhance EMT [8], provoke the transition of inflammation to cancer [31,34] and promote genomic instability, mainly by K-ras hyperactivation, which causes DSBs and DDR [58-60], (Figure 1).

3. Despite the relative abundance of let-7 in colon [5], no absolutely determined normal levels of let-7 expression in colorectum have been evaluated so far. Let-7 levels are quantified comparatively, in relation to its own expression in different situations, spatially [61] or temporally [11].

4. Let-7 isoforms aren't equally expressed both in normal and in the neoplastic colon. First, let-7-a, let-7-b [56], let-7-c [47], let-7-g and let-7-f [56] predominate in normal colonic epithelia. Second, let-7a and let-7-b are the prevalent isoforms found inside colorectal cancer cell lines [62]. Third, let-7-a [63,64], let-7-b [56,65], let-7-c/let-7-f [65] levels are preferentially repressed in colorectal neoplastic tissues compared to their paired normal ones.

5. Let-7's action is tissue-specific, disease-specific and cell-specific [46]. Acting upon stem cells, it forces them to differentiate [46], whereas it blocks the proliferation [47] and prevents the dedifferentiation of differentiated cells [46].

6. Let-7's action in a given cell of the colonic epithelium in a given time depends on the relative intracellular concentrations of its positive and negative regulators [8,13-17,34,39-44].

7. Let-7's expression in a given cell of the colonic epithelium in a given time depends on the relative luminal concentrations of environmental factors, as several dietary components upregulate (e.g. spinach [66]) or downregulate (e.g. polyamines [67]) mature let-7.

8. The antitumorigenic or oncogenic effect of let-7 depends on the stage of the tumorigenesis process. In the stage of colorectal adenoma and early carcinoma, *low let-7 levels are beneficial, i.e.* they halt the process: low let-7 levels induce apoptosis [35,37,43], induce oncogene-induced senescence [8,22], mainly via K-ras hyperactivation [18, 68] and enhance innate immunity against cancer progression [8, 36]. In late carcinoma stages, in the context of mutated p53, low let-7 inhibits apoptosis [8, 35,37] and increase stemness [46]. Thereby, in the advanced CRC stages, *low let-7 levels are deleterious.*

9. The antitumorigenic or tumorigenic effect of let-7 depends on the cell inside the growing colorectal tumor where it exerts its action. Increased aggressiveness of CRC is induced by *low let-7 levels* inside colorectal cancer cells (they drive stemness, EMT, invasion and metastasis [8, 45, 50, 69]) and by *high let-7 levels* inside stroma cells (they lead to diminished lymphocytic immunity against cancer cells [36]). The relative content of different cell types inside the tumor creates the dominant cellular environment and predetermines the overall contribution of let-7 in tumor's behavior.

10. Underexpressed let-7 accelerates every single stage of colorectal tumorigenesis [70]. In the incipient steps, low let-7 levels activate Wnt pathway, *via* the upregulation of β-catenin and c-myc [8,14,17,18,35,40]. Low let-7 levels activate K-ras and AKT in the stage of EGFR signaling [8, 17, 21-30] and TGF-β response in the next stage [8,10]. Following this, low let-7 levels permit p53 protein to over express [38]. Finally, low let-7 levels enhance the cancer-promoting EMT, as let-7 downregulates independently four EMT-favoring factors, *i.e.* TGF-β [8,10], HMGA2 [74], IL-6 [8, 34] and the acting cytoskeleton pathway [8] (Figure 1).

The Clinical Role of Let-7 as A Biomarker in Colorectal Cancer

Let-7 is very stable molecule in extreme pH values and in boiling, it is easy to be extracted from fresh or archival FFPE tissues, from stool or from blood/serum and can resist degradation over time. It is therefore candidate for being used as a biomarker [71,72]. Clinical research has given conflicting effects regarding the clinical value of let-7 in CRC. Concerning its diagnostic role, research works have shown either upregulated [73,74] or downregulated [75] let-7 isoforms levels in blood circulation of CRC patients compared to control group. Regarding prognosis, poor prognosis was correlated either to low let-7 levels [32,56] or to increased let-7 [37,76,77] in CRC tissues. Similarly, two investigators studying the influence of let-7 on survival of CRC patients, generated conclusions in opposite directions [78,79]. Last, regarding its predictive role to non-surgical therapy of CRC, most (but not all) studies have shown that high let-7 content in CRC tissues are correlated to radiosensitivity [8,55,80], to sensitivity to chemotherapeutics [5,54,55,78,79] and to improved prognosis after anti-EGFR antibodies (cetuximab) administration [22,32]. To make things complicated, mounting evidence suggests that

increased neoplastic let-7 levels, a presumably favorable predictive factor, which might drive doctors to recruit patients for advanced therapy, are downregulated during the corresponding therapeutic modality, *i.e.* CRC is capable to resist to radiotherapy [42,55,80] and to chemotherapy [81].

Finally, studies have not associated G-allele to CRC development [28]. Surprisingly, some works showed that it improves prognosis [25], whereas others demonstrated neutral effect [26]. Regarding its predictive role, a few works demonstrated that, despite its tumorigenic properties, G-allele was associated with improved prognosis both in naïve patients and in patients after chemotherapy or anti-EGFR antibodies therapy. Nevertheless, G-allele was not proved to be an independent predicting factor for CRC patients receiving anti-EGFR antibodies or other advanced therapy [21,22,25,27].

The Involvement of Let-7 in Colorectal Cancer Therapy

Let-7 holds dual properties; hence let-7-based therapy may be directed analogously. In tissues where let-7's tumor-promoting capabilities dominate, our therapeutic target is its inhibition. This is achieved by anti-sense oligonucleotides (ASOS), anti-miRNA oligonucleotides (AMOS) or microRNA sponges [10,82,83]. Nevertheless, let-7 is largely a tumor-suppressor in CRC; therefore our main pursue is to rocket up its expression or to restore its levels (gene therapy). To deliver let-7, adenoviral [10] or retroviral based [46] vectors have been proposed. Moreover, synthetic let-7 members, chemically modified RNA molecules that mimic mature isoforms' action have been invented and are delivered directly inside colorectal cancer tissue [46]. Indeed, delivering let-7 in CRC animal models has given encouraging results [46].

Unfortunately, a plethora of limitations and obstacles renders the manipulation of let-7 in CRC problematic. First, it is not feasible to decide whether to increase or lower let-7 levels in a given CRC, since low let-7 levels are not the characteristic of every single colorectal cancer tissue [46]. In spite the fact that the majority of studies have demonstrated reduction of CRC growth and invasion after restoration of let-7 levels, experimental data have given contradictory results [17,56,58]. As *in vivo* genetic studies of let-7 function are lacking [84], we are unaware about the isofrom(s) of let-7 that need restoration in a given colorectal neoplasm. The effects of exogenous let-7 on regulatory mechanisms of endogenous let-7 maturation and the ultimate effect of their interaction in the carcinogenesis process are largely obscure. Besides this, many critical genetic driver mutations are necessary for CRC development [85,86] and the isolated effect of a manipulator with central role, as let-7, upon them is largely unknown. Additionally, the possibility of immunization against exogenous let-7 has not been elucidated and the optimal delivery system is a matter of intense research [46]. The inhomogeneity of let-7 inside colorectal tissue renders almost impossible to achieve cellular target specificity, *i.e.* to restore let-7 levels exclusively in cancerous cells and not in stroma cells [10,12,83]. Moreover, since low let-7 levels are beneficial in early CRC stages, it is unknown whether the anti-tumor effect of increased let-7 levels is limited to advanced stages of tumorigenesis. Furthermore, synthesis and purification of therapeutic let-7 is quite difficult and let-7 restoration methods are not yet satisfactory [10]. The last hurdle to overcome is skin toxicity, attributed to virus-based let-7 delivery [32].

Let-7 and the Forthcoming Therapy of CRC: Prospects and Proposals

To put in a nutshell, let-7 is a strong manipulator of every single intermediate stage of colorectal tumorigenesis, inhibiting multiple crucial effectors. Apart from clarifying its diagnostic, prognostic and predictive role in CRC, future efforts should concentrate on let-7's main properties, *i.e.* the promotion of differentiation and the abolishment of stemness. Gene therapy, restoring let-7 levels or forcing its expression, may become a real fact, if we fully understand let-7's biology, regulation and interdependencies in normal and neoplastic colon before and after exogenous let-7 delivery, if we determine which genes are predominantly influenced when let-7 is delivered inside CRC tissue and if we shed light to the regulation and function of normal and CRC stem cells. Important issues should be resolved: ineffective delivery, difficulty in transducing large volume of cells in the tumors [46], inability to determine the "let-7 identity" of a given tumor and incapability to target let-7 delivery in a specific cell group inside the tumor. Furthermore, in order to use let-7 as an *in vivo* manipulator, the therapeutic indications (which CRC patients, which stage), the putative combination with other advanced therapies, the ultimate goals (remission of preexisting tumors *vs* prevention of tumor initiation *vs* halting neoplasia progression), specific details (which let-7 isoforms are necessary to deliver) and the contraindications of let-7 delivering strategy should become definite. Moreover, the undesired effects of let-7 administration (skin toxicity, induction of deleterious immune activation) must be determined and overcome. Finally, in order to be realistic, novel, let-7-based, therapeutic patents, like those recently invented in China and Australia [46] should focus on the field of CRC therapeutics.

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