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Let-7 Family miRNAs Represent Potential Broad-Spectrum Therapeutic Molecules for Human Cancer

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Abstract

miRNAs are a class of small non-coding RNAs that modulate gene expression. *Let-7* was first discovered in *Caenorhabditis elegans* and is one of the most extensively studied miRNAs. The human *let-7* family contains 13 miRNAs. The expression of these miRNAs is decreased in most human cancers and contributes to carcinogenesis and progression. Thus, the *let-7* family of miRNAs has attracted the attention of researchers in various fields. Exogenous *let-7* restoration has been confirmed to show antitumor efficacy in many human cancers. *Let-7* functions as a tumor suppressor by acting upon several multi-signaling pathways and multiple downstream target oncogenes that are involved in most human cancers. *Let-7* shows potential for modulation of chemoresistance and radiation sensitivity in human cancers. miRNAs in the *let-7* family represent potential broad-spectrum antitumor molecules for human cancer therapy, and miRNAs in this family have been studied intensively for their therapeutic potential. However, most previous studies have been limited to a single functional aspect or focused on a single effect in a particular type of cancer. Here, we review the latest research on *let-7* and discuss its potential value as a broad-spectrum antitumor molecule.

Keywords: *let-7*; Gene therapy; Cancer; Chemoresistance; Radiosensitivity

Abbreviations: *let-7*: Lethal-7; UTRs: Untranslated Regions; LCSs: *let-7* Complementary Sites; RCC: Clear Cell Renal Cell Carcinoma; Chol-let-7a: Cholesterol-Conjugated *let-7a* miRNA; 5-FU: Fluorouracil; *MDR1*: Multidrug Resistance 1; ER: Estrogen Receptor

Introduction

The lethal-7 (*let-7*) family of miRNAs is one of the most studied groups of miRNAs, and contains several prototypical miRNAs. *Let-7* was first identified in *Caenorhabditis elegans* (*C. elegans*), in which it was shown to exert a suppressive effect on let-60/Ras [1]. *Let-7* was subsequently identified as the first known miRNA and has since become the most-investigated human miRNA [2]. Thirteen members of the *let-7* family have been identified to date (*let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7d, let-7e, let-7f-1, let-7f-2, let-7g, let-7i, miR-98,* and *miR-202*); they have similar sequences and target a wide spectrum of genes [2,3]. *Let-7* family miRNAs are important for normal development, cell differentiation and are highly conserved in human tissues [4]. *Let-7* expression is commonly found to be down-regulated in human cancers, and this down-regulation contributes to carcinogenesis and progression [5,6]. Yu et al. infected BT-IC breast cancer cells with *let-7*-lentivirus, increased let-7 paralleled reduced H-Ras and HMGA2. Silencing H-Ras in a BT-IC-enriched cell line reduced self renewal but had no effect on differentiation, while silencing HMGA2 enhanced differentiation but did not affect self renewal [7]. This results showed that *let-7* regulated tumor cell self renewal and differenciation through different targets.

In addition, *let-7* miRNAs appear to play a role in the part of signal cascades, including Ras and NF-κB signaling cascades (Figure 1), which are involved in the suppression of invasion and metastasis of cancers, by interacting with upstream regulators and downstream *let-7* targets [8-10]. Iliopoulos et al. confirmed an epigenetic switch activates a positive feedback loop required for cell transformation, which involving NF-kB, Lin28, *let-7*, and IL-6 [11]. Normally, IL6-mediated activation of the STAT3 transcription factor is necessary for transformation, while *let-7* functions as suppressors directly inhibit IL-6 expression. When Src activation triggers a rapid inflammatory response mediated by NF-kB. This NF-kB directly activates Lin28 and rapidly reduces *let-7* levels, and resulting in higher levels of IL-6, and in turn IL-6 activates NF-kB, thereby completing a positive feedback loop for cell transformation. Choudhury and his colleagues confirmed *miR-21* and *let-7* control in an inversely related way in two major cancer pathways Ras and NF-κB [12]. Their study suggested that *let-7* is a direct negative regulator of the Ras gene family, and is proposed to repress the activation of NF-κB through down-regulation of Ras and IL-6; while *miR-21* suppresses multiple targets to activate Ras and enhances NF-κB activation through Pten-Akt and thereby increasing the activity of Akt.

These *let-7*–related signaling pathways are considered to be potential targets for therapeutic miRNAs [12,13-16]. *let-7* family miRNAs have been considered for use as potential biomarkers, as well as prognostic markers that can help predict cancer progression and response to treatment [17-25]. In addition, *let-7* miRNAs have emerged as a new class of potential therapeutic molecules for cancer and have been employed using a replacement strategy [26]. Restoration of *let-7* expression has been confirmed to be an effective therapy for human cancers [27]. Moreover, dysregulation of *let-7* has been confirmed

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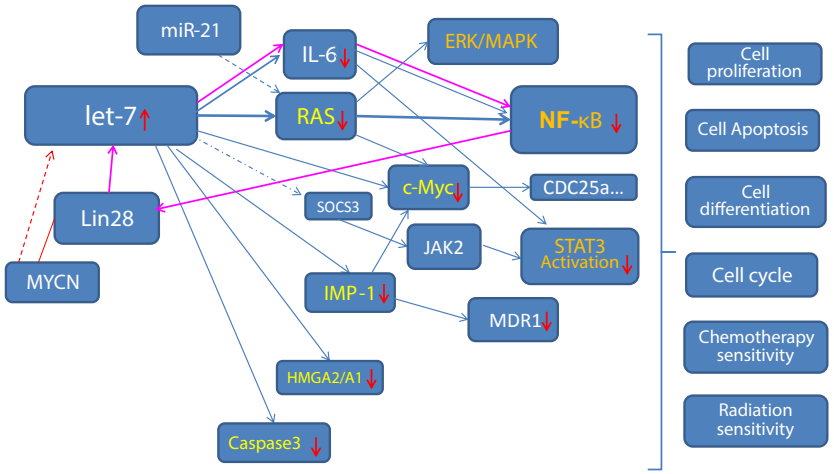

Figure 1: Summarize framework of important *let-7* related signaling pathways, and upstream regulators and downstream *let-7* targets.
Yellow words: Target genes; Orange words: Regulating signaling pathway
Purple arrows: Shows the positive feedback loop involving NF-kB, Lin28, *let-7*, and IL-6

involving with chemotherapy resistance and radiation sensitivity [8]. *let-7* family miRNAs are potential broad-spectrum antitumor molecules for human cancer.

Let-7 family miRNAs suppress the expression of a wide spectrum of target genes

The *let-7* pathway is evolutionarily ancient and plays vital roles in diverse biological processes. *Let-7* miRNAs regulate the expression of various downstream target genes such as human *Ras* (*K-Ras, N-Ras, H-Ras*), *HMGA2/A1*, *IMP-1*, *c-Myc*, and *caspase-3* [7,16,25,28-30]. *Let-7* miRNA was first confirmed to target the Ras signal pathway as a potential pan-Ras suppressor [1]. *Let-7* family members are direct and strong regulator of the RAS family. A study conducted in *C. elegans* predicted that the 3'- untranslated region (UTR) of human *ras* genes would contain multiple *let-7* complementary sites (LCS), which allow *let-7* to regulate *Ras* expression; it was later shown that the strong regulator *N-ras*, *K-ras*, and *H-ras* mRNAs contain 9, 8, and 3 potential LCSs, respectively [1]. Using comparative bioinformatics, 12 conserved *let-7*–regulated oncofetal genes were identified, including *HMGA2* and *IMP-1/CRD-BP* [31]. *IMP-1* and *HMGA2* are major miRNA targets. IMP-1 carries six putative LCS in its 3'-UTR, of which five are conserved among mammalian species. *IMP-1* is a direct target of *let-7* and promotes the growth and motility of tumor cells. Introduction of pre-*let-7g* or pre-*let-7d* caused down-regulation of IMP-1 protein expression in A549 cells, which express low endogenous levels of *let-7*. *Let-7* expression inhibits reexpression of IMP-1. Introduction of *let-7* into cells has a similar effect as the direct knockdown of IMP-1. Knockdown of *IMP-1* in MCF7 cells caused reduced expression of c-Myc. Whereas *let-7* can also affect the translation of c-Myc directly. *c-Myc* was also confirmed to be a target of let-7a via the predicted binding site in the 3'-UTRin Burkitt lymphoma cells [3]. A number of the identified putative *let-7* targeted cell cycle regulators are known to be c-Myc–regulated genes (i.e., CDK6, CDC25a). And there is a double-negative feedback loop between Myc and *let-7* miRNAs. Myc expression is inhibited by let-7d, whereas Myc inhibits some members of the *let-7* family sharing with Lin28. [30]. Computer-based sequence analysis showed that the first eight nucleotides from the 5' end of *let-7a* miRNA were complementary to nucleotides 153–159 of the caspase-3 3'-UTR [31]. Only *K-ras*, *h-ras*, and *n-ras* mutations are involved in 25–30% of all human cancers. Thus, *let-7* miRNAs represent potential broad-spectrum therapeutic molecules based on these multiple target genes.

Restoration of *let-7* expression shows therapeutic effects in human cancers

These target genes of *let-7* are important regulators that are involved in most human cancers. These results provide evidence for the potential broad-spectrum therapeutic use of *let-7* for human cancer, wherein *let-7* expression can be restored by exogenous miRNA replacement.

Studies have confirmed that the restoration of *let-7* miRNA expression significantly inhibit tumor growth and metastasis in human cancers *in vitro* and *in vivo*. Re-expression or overexpression of *let-7a* miRNA significantly inhibited cell proliferation or tumor growth in lung cancer and HepG2 and SMMC7721 hepatocellular carcinoma via suppression of K-Ras and c-Myc protein expression [29,32]. Another study reported that *let-7f* was capable of reducing cell growth of TPC-1 papillary thyroid carcinomas [33]. Further, the ectopic expression of *let-7g* in K-Ras–expressing murine lung cancer cells induced both cell cycle arrest and cell death. Significant growth reduction of both murine and human non-small cell lung (NSCL) tumors were also observed when *let-7g* was overexpressed using lentiviral vectors [34]. The plasmid vector pCMV-let-7g induced the re-expression of *let-7g* and inhibited tumor cell proliferation and migration through the K-Ras/HMGA2/Snail axis in MHCC97-H and HCCLM3 hepatocellular carcinoma [35]. *Let-7* miRNAs also showed antitumor effects in other human cancers, such as breast cancer and gastric cancer [36,37].

Activating *k-ras* mutations and K-Ras overexpression are present in nearly all pancreatic carcinomas. Blocking of the Ras pathway is a treatment strategy for pancreatic carcinoma [38]. However, less is known about the effect of restoration of *let-7* levels. *In vitro*, increased *let-7* levels result in strong inhibition of cell proliferation, but *in vivo* they fail to impede tumor progression through vector-induced stable *let-7* miRNA overexpression, as well as restoration through intratumoral gene transfer of *let-7* miRNA [39]. There are three possible explanations for this. The dysregulation of *let-7* miRNAs may differ in different types

of cancers; thus a *let-7* member may display different functions in different cancers [3,17,32,33,40]. The study conducted in *C. elegans* [1] predicted that although the 3'-UTRs of human *k-ras* mRNAs contain 8 potential LCSs, However, only 1 LCS is conserved among mammalian species. Further, the highly conserved let-7 miRNAs have been shown to have suppressive effects in cancers with fewer mutated *ras* oncogenes but overexpression of Ras proteins, such as in hepatocellular carcinoma [35,38,41]. We verified that *let-7a* targets wild-type and mutated *ras* genes in humans through luciferase activity reporting analysis system. Our results showed that targeting of *K-ras* yielded a >30% reduction, whereas targeting of wild-type *N-ras* produced a 50% decrease in luciferase activity compared to the control, and no significant difference was observed with targeting of wild-type *H-ras* and mutated *K-ras* and *N-ras* (Figure 2). This result is consistent with previous studies [1,35,38,41]. The intratumoral transfer of molecules to other tumors may provide another explanation. Our previous study suggested that cholesterol-conjugated *let-7a* miRNA (*Chol-let-7a*) shows stronger inhibitory effects when administered systemically than when delivered through local injection [32,42,43].

The restoration of *let-7* miRNAs not only inhibits tumor growth and metastasis, but also promotes cell apoptosis and cell differentiation, which also exert therapeutic effects against human cancers [32,33]. *Let-7a* restoration regulates cell death by directly targeting caspase-3 and BCL-XL, or by silencing Aurora-B [27,41,44,45]. Chol-let-7a miRNAs promote HCC cell apoptosis *in vitro* and reverse the orthotopic tumor cell phenotype, with no significant atypia in most areas after Chol-let-7a systemic therapy [32].

These results confirmed that restoration of *let-7* miRNA expression can inhibit tumor growth and metastasis in different type of human cancers. *Let-7* miRNAs are therefore broad-spectrum antitumor molecules that can be used in human cancer therapy.

The potential of *let-7* miRNAs to modulate the drug resistance and radio sensitivity of human cancers

Drug resistance remains an important problem in treatment of cancer, especially in advanced and recurrent cases. Identification of the factors regulating drug resistance will help in development of strategies to resolve this problem.

Previous studies have reported that the dysregulation of *let-7* expression in human cancers is associated with chemoresistance [46-50]. Lower *let-7a* expression was associated with epirubicin resistance in primary breast tumors, wherein upregulation of *let-7a* expression sensitized resistant breast tumor cells to epirubicin *in vitro*. [46]. *Let-7b* and *let-7c* were significantly downregulated in clear cell renal cell carcinoma (RCC) tissues and the dysregulation of *let-7b* and *let-7c* contributed to the chemoresistance of RCC cells to fluorouracil (5-FU) by downregulating Akt2. Transfection of *let-7b* or *let-7c* combined with 5-FU inhibited proliferation and potentiated the antitumor effects

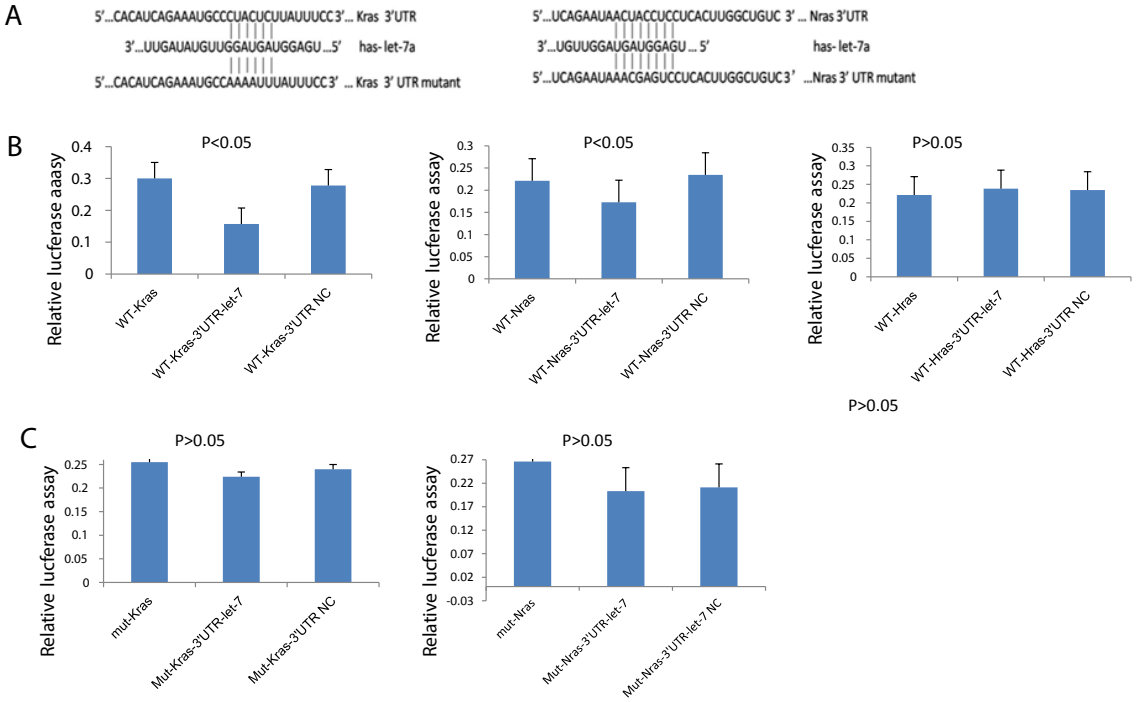

Figure 2: Let-7a target human Ras by luciferase reporter assays.

293A cells(2×10^4) were incubated in 24-well plates for 24h and transfected with Lipofectamine 2000 (Invitrogen) and 100 ng pmiR-Glo-3' UTR reporter vector including binding sites for let-7a (Promega, Madison, USA) and let-7a mimics or a negative control. After 24h, cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each well. Experiments were repeated 3 times.

(A) LCRs of human wild-type *K-ras,N-ras* (3' UTR) to *let-7a;* LCRs loci in mutated human *K-ras,N-ras*

(B) Luciferase reporter assays of wild-type human *K-ras,N-ras*

(C) Luciferase reporter assays of wild-type human *K-ras,N-ras*

The results represent the mean ± SEM. All data analyses were performed with SPSS 17.0 software (IBM, Inc., Armonk, NY, USA). Student's t-test was used for statistical comparisons between groups. $p<0.05$ was considered to be statistically significant.

of 5-FU at tolerable concentrations, and then enhanced sensitivity to 5-FU by affecting the apoptotic pathway [51]. In vitro results showed that *let-7g* miRNA contributed to an increase in 5-FU-induced cell cycle inhibition in human hepatoma cells and sensitized the cells to 5-FU, leading to increased effectiveness of the drug in the treatment of hepatoma [52]. Patients with relapsed ovarian cancer tested before and after chemotherapy also showed down-regulation of *let-7g* and upregulation of IMP-1 and multidrug resistance 1 (MDR1). Moreover, the introduction of *let-7g* into ADR-RES ovarian cancer cells reduced the expression of both IMP-1 and MDR1 and rendered the cells more sensitive to treatment with taxol or vinblastine [53].

Dysregulation of *let-7* expression in human cancers is associated with radiation resistance. In A549 lung carcinoma cells and ASPC1 pancreatic cancer cells possessing a *k-ras* mutation, *let-7a* overexpression decreased K-Ras expression and radiosensitized the A549 cells. Inhibition of Lin28, a repressor of *let-7*, attenuated K-Ras expression and radiosensitized A549 and ASPC1 cells [54]. These results confirmed that dysregulation of *let-7* is associated with radiation resistance.

In breast cancer cells, *let-7a* and *let-7b* were dramatically decreased. Following pre-*Let-7a* miRNA transfection, overexpression of *let-7* dramatically enhanced the sensitivity of SK-BR-3 clone (S1) cells to radiation, which suggested that downregulation of *let-7* miRNA could be one of the mechanisms underlying Lin28-induced radioresistance in breast cancer cells [40,41,55]. The association of Lin28, an inhibitor of *let-7* miRNA, with paclitaxel resistance and radiation resistance was also confirmed in breast cancer cells [55,56]. Lv et al. confirmed that the T47D cancer cell line, which highly expresses Lin28, is more resistant to paclitaxel than the MCF-7, Bcap-37, or SK-BR-3 cancer cell lines, which show low-level expression of Lin28 [55]. T47D cells also showed increased sensitivity to paclitaxel treatment after knock-down of Lin28. Combinatorial treatment for NSCLC with *let-7b* and *miR-34a* resulted in the strongest synergistic enhancement of the efficacy of erlotinib. In addition, transfection with *let-7b* is known to reverse drug sensitivity in chemotherapy-resistant SGC7901/DDP and SGC7901/VCR gastric cancer cells by targeting the downregulation of c-Myc [57]. These results indicate that let-7 miRNAs can effectively reverse drug resistance and can be used as adjuvant therapeutics for the treatment of human cancers to modulate chemotherapy and radiation resistance [58,59].

Broad-spectrum antitumor molecules, advantages, and challenges

Let-7 miRNAs represent a type of potential broad-spectrum therapeutic molecules, based on the mechanism of miRNAs differs from that of siRNAs, which mediate sequence-specific cleavage of nascent mRNAs [60]. This is an advantage of all miRNAs, not *let-7* alone. Unlike siRNAs, miRNAs require only partial complementarity to the 3'-UTR of their target mRNA to regulate their multiple targets [60]. These we have discussed above. Thus, a perfect match is not necessary for miRNA function. When used as a drug, there is therefore no need to design special miRNAs to target different oncogenes or different cancers. We have recently confirmed that vector-based *let-7* dsRNA suppresses neuroblastoma tumor growth by acting against multiple targets, including N-Ras, K-Ras, c-Myc, and HMGA2 (data not shown). Although the vector used is a vector-based siRNA construct of the type used to produce dsRNAs (siRNAs) of interest, upregulation of *let-7a* only successfully suppresses the N-Ras, K-Ras, c-Myc, and HMGA2 proteins, but does not down-regulate mRNA levels of the related oncogenes (data not shown). This result suggests that the vector-based *let-7a* expression also function as miRNAs, but not siRNAs. Mechanism of the molecule may primarily depend on its sequence but not the method to produce.

Broad-spectrum drugs, compared with relatively specific drugs, have advantages in terms of reduced research and development costs, and benefit more patients. In addition, because cancer is a multifactorial disease, *let-7* should be more effective than single-target drugs. Members of the *let-7* family can be used as effective antitumor molecules either singly or in combination. *Let-7* can be combined with other RNAi molecules, such as siRNA or other miRNAs, as part of a small-molecule cocktail therapy, which may create a new strategy for cancer treatment. In addition, *let-7* can be combined with existing chemotherapy drugs, targeted biological drugs, and clinical treatment methods such as radiotherapy, to increase sensitivity to drugs and radiation therapy. In addition, let-7 may delay drug resistance to chemotherapy and radiotherapy.

However, multi-target drugs may produce more adverse off-target and side effects. During assessment of the potential of *let-7* as a therapeutic molecule, its off-target effects should be considered. We examined the off-target effects of cholesterol conjugated *let-7a* (*Chol-let-7a*) to liver and kidney in nude mice when it is used via systemic delivery method. It is encouraging that the results suggested that *Chol-let-7a* induced only mild off-target effects, including non-specific reaction changes in the liver and kidney following systemic administration for 5 weeks (data not shown). *Let-7a* levels were significantly increased in *Chol-let-7a*–treated xenografts, whereas *let-7a* levels were still much lower in orthotopic tumors than in normal control livers. *Let-7a* abundance in *Chol-let-7a*–treated liver tissues did not differ from that of normal control mice. Thus, the off-target effects of additional *Chol-let-7a* appear to be slight. We think that most of the reactive features may result from the delivery system and the tumor self.

In addition, off-target effects could be avoided by developing a targeted delivery system and by choosing patient-specific routes of administration according to tumor type. For example, local injection could be used in thyroid cancer, as cholesterol-conjugated *let-7* miRNA has been confirmed to be a potential useful liver-target carrier for systemic therapy of hepatocellular carcinoma and liver metastatic malignant tumors originating from pancreatic carcinoma, colorectal carcinoma, or lung cancer.

Hurdles and limitations in the use of let-7 miRNAs as drugs

Let-7 miRNAs have shown potential therapeutic effects in human cancers, and represent broad-spectrum antitumor molecules. A few preclinical and clinical studies using members of the *let-7* family are currently in progress [32,61-63]. However, much additional research is required before *let-7* miRNAs can be used as drugs in clinical settings, especially for systemic therapy.

miRNA-based approaches has moved toward clinical trials base on many preclinical studies that have produced promising results. As well as clinical trials that are being planned or are underway [27]. Although the results and conclusions of the clinical trials will provide significant value for miRNA therapy, including let-7. However, a major hurdle to the clinical use of *let-7* family miRNAs for systemic therapy is the lack of an effective, non-toxic carrier; this is also true for several other miRNAs known to have therapeutic effects.

The delivery of exogenous miRNAs is based on the use of viral and non-viral vehicles [64-66]. Viral vehicles are not preferred as they

are known to cause death due to severe liver cytotoxicity in mice [67]. Certain non-viral vehicles have been used for miRNA transfer in recent years. Chemical modification, such as cholesterol labeling and the use of nanoparticle delivery vehicles, has been successful when used for the delivery of miRNAs in vivo. However, it is difficult to choose low-toxicity nanoparticles for clinical use based on the available experimental models [68,69]. As shown in a few studies primarily based on *in vitro* cell viability or as observed in preclinical models, the toxicity of nanoparticles has two primary causes. First, silver nanoparticles show potential inflammatory effects, heat shock, and diverse cellular effects; second, nanoparticles can potentially damage the genetic material, as they can cross cell membranes and reach the nucleus [70,71].

Synthetic *miR-34a* and *let-7* mimics have been successfully delivered to target lung tissues using neutral lipid emulsions; they were found to cause a reduction in lung tumors in mice [70,72]. Zhang et al. designed a hepatocyte-targeting ligand to increase the efficiency of targeted delivery of anti-*miR-155* [71,73]. Our results have shown that *Chol-let-7a* miRNA targets liver tissues and orthotopic tumors while causing mild inflammation and cellular cytotoxicity in the livers and kidneys of nude mice. In addition, a significant upregulation of *let-7a* was observed in tumor tissues, but *let-7a* levels remained much lower than those in normal liver cells and tissues (data not shown). These targeted miRNA delivery methods resulted in strong antitumor effects with few off-target effects, and show potential for use as delivery systems in cancer therapy. However, the safe and effective delivery of *let-7* miRNAs into solid tumors remains a challenge. Additional studies, with a particular focus on exploring off-target effects induced by both the delivery system and *let-7* miRNA overexpression, are needed.

Conclusions

Dysfunction of *let-7* miRNAs is implicated in most human cancers. *Let-7* miRNAs are involved in tumor growth, and resistance to chemotherapy and radiation therapy. *Let-7* family miRNAs represent broad-spectrum antitumor molecules for cancer therapy.

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