Chitosan Nanoparticle as a Delivery System of miRNA 217 for Suppressing Hepatocellular Carcinoma Progressivity by Targeting AEG-1/P53

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Abstract: MicroRNA, especially miRNA-217, has important role in development of Hepatocellular Carcinoma (HCC) through its relation with astrocyte elevated gene 1 (AEG-1) who modulates several signaling arrays. Delivery systems could be crucial factors for successful gene therapy. We investigated effects of chitosan nanoparticles as delivery system of miRNA-217 for targeting AEG-1 in HCC. Chitosan nanoparticles were prepared using ionic gelation methods. Entrapment efficiency was obtained using a NanoDrop spectrophotometer. Mimic miRNA-217 encapsulated by chitosan nanoparticles were transfected in HCC cell line HepG2. Viability test was conducted by using MTT Assay. The dosages of miRNA-217 were ¼, ½ and 1 IC50. A real-time polymerase chain reaction determined miRNA-217 and mRNA relative expressions. Independent T-tests were used to analyze the parameter differences. Results showed that chitosan nanoparticles could encapsulate miR-217 with 92.9% entrapment efficiency. miR-217 was successfully delivered and significantly increase the endogenous expression of miRNA-217 in HepG2 cells compared to controls. It mediated significant cell inhibition in chitosan nanoparticles group compared to naked miRNA. The expression of mRNA AEG-1 was decreased significantly compared to controls. The increased expression of miRNA-217 was negatively correlated to AEG expression. Chitosan nanoparticles of miRNA-217 may suppress cell line progressivity via targeting AEG-1.

1 INTRODUCTION

Hepatocellular carcinoma causes the third-highest cancer-related mortalities with the least survival years worldwide (Jariwala et al., 2015). The life expectancy is less than six months from diagnosis (Torre et al., 2015; Xie et al., 2017; Jia et al., 2018). Furthermore, most patients with HCC are diagnosed late because of the asymptomatic course of the disease. The therapeutic effects of several treatments of HCC are still limited. Since the underlying mechanisms of the formation of HCC are still elusive, novel therapeutic strategies are needed for this aggressive malignant tumor (Jia et al., 2018).

MicroRNA (miRNA) is known to have an important role in the regulation of gene expression. Lu et al. revealed that all cancers have different miRNA expression profiles than normal tissues (Lu et al., 2005; Santos-carballal, 2018). Downregulated miRNAs serve as a tumor suppressor miRNA...
regarding HCC development and progression (Jia et al., 2018). One of the tumor suppressor miRNAs is miR-217, which is downregulated in patients with HCC (Jariwala et al., 2015).

The research reported several oncogenic pathways are regulated by astrocyte elevated gene 1 (AEG-1). These are Akt, nuclear factor-kB (NF-kB), Wnt/β-catenin, and mitogen-activated protein kinase (MAPK) pathways. Additionally, the upregulation of AEG-1 expression can promote cell proliferation and anchorage-unrestrained growth ability in HCC (Meng et al., 2012; Shi and Wang, 2015). Other previous studies showed overexpression of mRNA and protein AEG-1 levels in most patients with HCC, associated with cancer progression and aggressive metastatic stage (Yoo et al., 2009; Sarkar, 2013; Robertson et al., 2014).

Recently, mRNA delivery systems were explored to verify the most appropriate method to transfect mRNA into target cells. However, nanoparticles are the preferred method because of their potential advantages (Phua et al., 2013). Nanoparticles can protect the mRNA, which is susceptible to nuclease degradation and facilitate uptake leading to targeted genes (Phua et al., 2013; Glackin et al., 2018). Cationic-based nanoparticles can interact with a negatively charged nucleic acid to form nanocomplexes. One of the polycations of nanoparticles is chitosan. This polymer has been widely studied due to its biodegradability and low toxicity (Esquivel et al., 2015). Several methods can be applied to prepare chitosan-based nanoparticles, and the simplest one is ionotropic gelation (Gennari et al., 2019). This method's key lies in the strong electrostatic interaction between polymers and crosslinker agents (Esquivel et al., 2015; Prasetyo et al., 2019).

As previously known, viral vectors and lipofectamine play a pivotal role in drug delivery for gene therapeutics. In contrast, viral vectors' disadvantages include significant safety issues and immunogenic responses (Santos-carballal, 2018; Guo and Huang, 2012). In terms of cytotoxicity, lipofectamine has serious toxicity in cell viability (Mukerjee et al., 2011). Herein, we report the development of a novel miRNA-217 based chitosan nanoparticles preparation, which can be employed for drug delivery (Lee et al., 2011; Khan et al., 2019). To elucidate the intracellular processing of miRNA 217, we investigated chitosan nanoparticles’ effect delivery system of miRNA-217 for targeting AEG-1 in HCC.

2 MATERIALS AND METHODS

2.1 Materials

Molecular medium-weight chitosans were obtained from Sigma Aldrich (St. Louis, MO). Sodium tripolyphosphate was purchased at LPPT Universitas Gadjah Mada. The human carcinoma cell line HepG2 was obtained from BPPT (Jakarta, Indonesia). It was maintained in high glucose DMEM supplemented with 10% fetal bovine serum (Massachusetts, USA), 1% penicillin-streptomycin (Massachusetts, USA), and 0.5% amphotericin (Massachusetts, USA) at 37°C with 5% CO2. miRNeasy Mini Kit, miRCURY LNA® RT kit, SYBR green PCR kit and SensiFASTTM SYBR® were purchased from Qiagen (USA).

2.2 Chitosan-Nanoparticle based miRNA-217 Formulation

The chitosan medium molecular weight was dissolved into 1% acetic acid. The solution was vigorously stirred using a magnetic stirrer for 4 hours. The pH of the solution had been adjusted to 5.5 while NaOH 1 M was added. The solution 1% chitosan was added with acetate buffer pH 5 to generate 0.2% chitosan solution. Preparation of chitosan nanoparticles was done with ionic gelation methods. It was obtained by mixing 0.2% chitosan and sodium tripolyphosphate (5:1) and incubating for 5 minutes at room temperature. Then, 150 μL mimic miR-217 was conjugated into 150 μL of chitosan nanoparticle solution and then incubated for 20 minutes at room temperature (Ysrafil et al., 2020).

2.3 Entrapment Efficiency

miR-217, which was formulated by chitosan nanoparticles, were centrifuged for 15 minutes at a speed of 13,000 g. The absorbance of supernatants was measured by using NANO-Quant. Determination of efficient entrapment was calculated using the following equation:

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EE\% = \frac{\text{Encapsulated miRNA} - \text{free miRNA}}{\text{Encapsulated miRNA}} \times 100\% \quad (1)
\]

2.4 Determination of Cell Viability

MTT assay method was used to test HepG2 Line cells' cytotoxic activity as much as 6 x 10^3 HepG2 cell lines were planted on a 96-well plate and incubated at...
37°C, 5% CO₂ for 24 hours. Then, the media was discharged from the plate and cleaned using PBS. As much as 100 μL/well of prepared chitosan nanoparticles mixed with serum DMEM free media were put into each well and incubated at 37°C and 5% CO₂ for 24 hours. The following day, each well was discharged and filled with 100 μL MTT 0.5 mg/ml and then incubated for 4 hours at 37°C and 5% CO₂. 100 μL SDS 10% were added to stop the reaction, dissolve formazan crystals, and then incubated overnight. Each well's absorbance was determined by using a Micro Plate Reader (Bio-Rad Model 680 XR) (Ysrafil et al., 2020).

2.5 Cell Transfection and mRNA Expression Assay

Cells (5x10⁵ per well) were plated in 6-well plates and incubated at 37°C and 5% CO₂ for 24 hours. After removing the media, each well plate was filled with 750 μL prepared chitosan nanoparticles (mixed with free media serum) and incubated at 370°C and 5% CO₂ for 24 hours. According to the kit's instruction manual, total RNA was isolated using the miRNeasy Mini Kit (Qiagen). A nanodrop determined the concentration of RNA. Then, cDNA was synthesized using the miRCURY LNA™ RT kit (Qiagen). Quantification of miR-217 was determined by quantitative polymerase chain reaction (PCR) using miRCURY LNA™ and SYBR green PCR kit. The sequence of miRNA and references were: 5’UACUGCAUCAGGAACUGAUUGGA 3’ and 5’UAGCAGCACGUAAAUAUUGGCG 3’, respectively. Meanwhile, the quantification of AEG-1 mRNA expression was measured by qPCR using SensiFASTTM SYBR®. The primers for AEG-1 and beta-actin mRNA were as follows for forward: 5’TGACTTCAACAGCGACACCCA 3’; reverse: 5’CACCCTGTGCTGTAGCCAAA-3’ and forward: 5’GGGAATTTCAAACTGGAAACGGTG GAAGG3’; and reverse: 5’GGAAGCTTATCACAGTCCCTGCGCCACA-3, respectively. The Biorad CFX 96 C.1000 quantitative PCR machine was used to quantify measurements of all gene transcriptions. The experiments were performed in triplicate. Relative gene expressions were analyzed using the 2⁻ΔΔCT methods, and the results were expressed as the fold change.

2.6 Statistical Analysis

All measurements were presented in mean ± standard deviation (SD). To determine the significant difference between groups, independent t-tests were performed in each group. Spearman/ Pearson correlation analysis was also done to find the relationship between miRNA-217 and AEG-1 mRNA. All data were analyzed using SPSS 22 software (IBM Corp., Chicago), and graphics were presented by GraphPad Prism 7. Statistical significance was set at P < 0.05.

3 RESULTS

The Entrapment efficiency NPs-Ch-miR-17 was presented in fig.1. The absorption efficiency of NPs-Ch-miR-17 was 92.19%. This percentage showed the amount of miRNA 217, which is in the nanochitosan matrix.

3.1 Viability Cells

The cytotoxicity effect of nanoparticles mimic miRNA 217 in the HepG2 -HCC cell line was measured to determine viability cells test. Variety of dosage concentrations of NPCs mimic miRNA 217 had were correlated to the presentation of cancer cell inhibition up to IC50 value. The IC50 value of this study was 120 nM. The result of the viability cells assay had been shown in figure 2.
3.2 In Silico Target Prediction

Figure 3 showed the results of in silico target prediction of miRNA 217. It showed AEG 1 had been a target of miRNA 217 at 833-856 base with ΔG = 20.5 kcal/mol. Based on the result, miRNA217 is a tumor suppressor miRNA regulating oncogenic post transcription gene AEG-1.

The independent t-test was conducted to whether there were significant differences between the 30 nM group (1/4 IC50), the 60 nM group (1/2 IC50), the 120 nM group (IC50) and the control group. These findings resulted in a significant difference between treatment groups compared to the control group with a p-value <0.005.

Figure 2: Viability assays to analyze inhibition of Proliferation of chitosan nanoparticle of mimic MiR-217 to HepG2 cell line (n=3). It is presented in mean ± SD. *P < 0.05.

Figure 4: Relative of expression endogenous miRNA 217 (A) and mRNA AEG-1 (B) HepG2 cell line post treatment nanoparticles mimic miRNA 217 in several doses which were measured by qPCR

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Meanwhile, the highest level of difference was found in the group with a dose of 120 nM with a p-value < 0.001. To determine whether there was a correlation between miRNA-217 expression and AEG-1 in each treatment group, the Pearson test was performed and obtained r and p values of -0.870 and 0.0001, respectively, which showed there was a very strong negative correlation between relative expressions of miRNA 217 and AEG-1 mRNA (p < 0.05).

4 DISCUSSION

Mechanism of microRNA-217 encapsulation through the ionic interaction between negative charge of the (-NH3+) group miRNA and positive charge of tripolyphosphate of crosslinker. Indeed, the concentration of chitosan affects entrapment efficiency. The low chitosan concentration's low viscosity might cause effectively penetrating miRNA into the polymer matrix (Csaba et al., 2009; Ysrafil et al., 2020). In this study concentration of chitosan was 0.2%. Like previous research, this result showed that drug encapsulation efficiency prepared by the ionic interaction method was more than 90% (Agnihotri et al., 2004).

The mitochondrial dehydrogenase assay was used to determine cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. Indication of mimic microRNA has effectively entered the HepG2 cell is the percentage of cell growth in the treated group led to initiate cancer cell death (Ysrafil et al., 2020). Furthermore, the expression of miRNA 217 was upregulated in the treated group compared to controls.

The addition of a positive charge from the cationic polymers into miRNA causes agarose cells' oligonucleotide migration ability. As a delivery system, chitosan nanoparticles will protect miRNA from the degradation of nucleases in HepG2 cells. Inside these cells, chitosan nanoparticles will interact with lysozyme, an enzyme that can degrade chitosan after cellular uptake occurs. The enzyme hydrolyzes the glycoside bonds in chitosan's chemical structure, causing the release of miRNA in the cytosol leading to diminishing the targeted gene's expression (Freier et al., 2005).

The study conducted by Zhang et al. in 2017 used lipofectamine 2000 as a miRNA 217 mimic transfection agent. The study used a dose of 100 nM with an incubation period of 36 days resulting in upregulation of miRNA-217 with a relative expression of 2.6-2.7 times higher compared to controls and downregulation of MTDH with expression values which were relatively 0.3-0.4 times lower compared to controls (Zhang et al., 2017). These results are almost the same as in this study using the chitosan nanoparticle transfection agent. However, using a lipofectamine drug delivery system certainly has a negative effect, namely high cytotoxicity (Wang et al., 2018). Cytotoxicity studies are one of the main concerns for the selection of transfection reagents. Generally, transfection reagent toxicity has a positive correlation with transfection efficacy. However, both are influenced by the type of cell (Wang et al., 2018).

One of the target genes of miRNA 217 is AEG-1. This study follows previous research conducted by Zhang et al. in 2016, which explains that miRNA 217 can inhibit HCC cancer cell proliferation by targeting MTDH. The finding showed miRNA 217 could suppress AEG-1 of mRNA and protein expression (Zhang et al., 2017). Another study stated that mir-30a-5p could reduce mRNA expression and MTDH protein (Li et al., 2015).
Also, the activation of various signaling pathways, such as PI3K/Akt, NF-κB, and Wnt/β-catenin pathways, is regulated by AEG-1 (Yang et al., 2018). Increased expression of AEG-1 causes increased expression of genes that support malignancy (He et al., 2015). Hence, it interacts with NFkB resulting in growth, survival and invasion of cancer cells. Simultaneously, tumor progression and proangiogenesis through the PI3K/AKT pathway and the Wnt/β-catenin pathway MAPK cause changes to epithelial-mesenchymal transition for metastasis (Dhiman et al., 2019). The NFkB pathway also becomes active in the presence of AEG-1 phosphorylation. Increased AEG-1 expression will activate inflammation regulated by NFκB, supporting activation inflammation regulated by NFκB, supporting tumor development in HCC (Sarkar et al., 2008; Emdad et al., 2016). Another study also states that AEG-1 overexpression can suppress PTEN protein expression (Li et al., 2015). Meanwhile, Wen fang li et al. revealed that apoptosis-related protein expression, notably PTEN and p53, had been regulated by AEG-1 (Li et al., 2015).

These findings suggested that deletion of AEG-1 might increase p53 expression by upregulating PTEN. As a tumor suppressor, p53 can initiate cell death and suppress cell proliferation (Li et al., 2015; Kruiswijk, Labuschagne and Vousden, 2015). Another study revealed that the absence of AEG-1 induces apoptosis of hepatocytes, hinder mutated transformation. Overexpression of AEG-1 encourages the tumorigenic process to maintain various stress (Robertson et al., 2018). Increased cytoplasmic AEG-1 expression acts as an RNA binding protein that can induce chemoresistance. It is of clinical significance to restore anti-cancer therapy’s sensitivity (Meng et al., 2012). This study strongly suggested developing AEG-1-targeted as a promising therapeutic strategy. Indeed it deserves further investigation.

5 CONCLUSION

We concluded that nanoparticle chitosan could be used as a delivery system for targeted therapy. In addition, miRNA 217 can suppress hepatocellular carcinoma progressivity by targeting AEG-1/p53.

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CONFLICT OF INTEREST

There is no conflict of interest in this research.

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