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Lentiviral shRNA Construction in Gene Therapy for Knock Down CYPIA1, and YWHAZ gene in Lung Cancer

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Abstract

Lung cancer is the top 10 deasses causing death globally. It is caused by CYP1A1 and YWHAZ genes expressed in the lungs. One of the solutions offered for lung cancer sufferers is gene therapy. It is focused on the knock-down of expressed CYP1A1 and YWHAZ genes. It can be using shRNA transfected into lung cells by lentivirus. The lentivirus has envelope that can be modified to be lentivirus pseudotyping by using glycoprotein filovirus, one of which is ebolavirus. Lentivirus pseudotyping can recognize certain cells. Especially, Ebolavirus can recognize lung cells. The lentiviral gene is designed to carry the gene of interest (shRNA) but no the infectious gene. The shRNA gene transfused into lung cancer cells will recognize and complement the specific target mRNA sites of the CYP1A1 and YWHAZ genes. After that, shRNA will cut the mRNA which should be expressed into a cancer protein. Unexpressed mRNA into protein causes cancer cell growth inhibited. Thus, this gene therapy solution is expected to be able to reduce the rate of death caused by lung cancer.

Keywords: Lung cancer, CYP1A1 and YWHAZ genes, gene therapy, lentiviral, shRNA

A. Introduction

Lung cancer or lung carcinoma is a malignant lung tumor caused by uncontrolled cell growth of lung tissue (Mustafa, et al. 2016). Based on data presented by WHO (2019), lung cancer is a disease that is in the top 10 causes of death globally, to be precise it is in the 6th top rank after ischaemic heart disease, stroke, chronic obstructive pulmonary disease, lower respiratory infections, and neonatal conditions. The risk factors include smoking both active and passive, exposure to radon gas, asbestos, air pollution, and genetic factors. Smoking is the main cause of lung cancer (Mustafa, et al., 2016). Smoking can cause CYP1A1 and YWHAZ genes expressed in the lungs. One solution that can be applied to reduce death cases of lung cancer is the use of gene therapy.

Gene therapy for lung cancer is focused on the knock-down of expressed CYP1A1 and YWHAZ genes. They are important gene causing growth lung cell abnormally. CYP1A1 converts tobacco combustion products containing PAHs into reactive metabolites that may be involved in the initiation of carcinogenesis. Meanwhile, YWHAZ is one of the hub genes with the highest level of presence. The encoded proteins are involved in many vital cellular processes such as

signal transduction, metabolism, cycle regulation, and apoptosis. YWHAZ is a potential regulator of β-catenin function, which is a central effector of Wnt signaling in tumorigenesis and metastasis. Therefore, both are important gene causing of lung cancer.

The way to knock down the gene is by using shRNA which is transfected into lung cells using a lentivirus. The envelope of Lentiviruses was modified using glycoprotein filovirus, one of which is ebolavirus (Cronin et al., 2005). This lentivirus pseudo-typing can recognize certain cells. Ebolavirus has ability to recognize lung cells. The lentivirus gene is designed to carry the gene of interest (shRNA) but no infectious gene. The shRNA gene that has been transfused into lung cancer cells will recognize and complement with the specific target mRNA sites of the CYP1A1 and YWHAZ genes. After that, it will be processed into siRNA. siRNA will cut the targeted mRNA which should be expressed into a cancer protein. Thus, targeted mRNA will not be expressed into protein. The growth of cancer cell will be inhibited. Based on this background and line of thinking, the aim of this research idea is to knock down the CYP1A1 and YWHAZ genes for lung cancer sufferers.

B. Literature Review

1. Lung cancer

Lung cancer is a type of cancer occurring in the lungs. Cancer starts when cells in the body begin to grow out of control. There are two types of lung cancer. First, it starts from part of the lung cell (primary lung cancer) and it starts from other cells but spread to the lung cell (secondary lung cancer). Lung cancers typically start in the cells lining the bronchi and parts of the lung such as the bronchioles or alveoli (cancer.org). Lung cancer is in the top 10 as a disease that causes death globally, which is in the 6th top rank after others (WHO, 2019). It is more common in men, and incidence increases with age (Neal, 2019).

Worldwide, lung disease is mostly caused by smoking, which is about three-quarters. Another was due to other risk factors, like exposure to radon, and air pollution (Neal, 2019). Smoking can increase the CYP1A1 and YWHAZ gene expression. They are the genes having important roles in causing lung cancer.

2. CYP1AI and YWHAZ genes

CYP1A1 (Cytochrome P450 Family 1 Subfamily A Member 1) is a Protein Coding gene, located at 15q22-q24, comprises of seven exons and six introns and spans 5,810 base pairs. It encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins localizes to the endoplasmic reticulum. It is associated with Aryl Hydrocarbon Hydroxylase Inducibility (AHHI) and Gastroschisis (genecards.org and Najm et al., 2013). AHHI is related to *lung cancer* and *aropharynx cancer* (Malacard.org). It also has a good association with polycyclic aromatic hydrocarbons (PAHs). Association of CYP1A1, AHHI, and PAHs can cause a lung cancer (Kouri et al, 1974). PAHs, some of which are found in cigarette smoke (genecard.org), especially in tobacco combustion products (Cao et al., 2015). When the human consumes smoking, tobacco combustion products will contain PAHs. PAHs will enter to the human body and be changed to reactive metabolites. It will induce AHHI (Kouri et al, 1974). AHHI has associated with CYP1A1. In human lungs, high CYP1A1 expression is associated with an increased risk of lung cancer (Cao et al., 2015).

Meanwhile, YWHAZ is one of the hub genes with the highest level of presence. The encoded proteins are involved in many vital cellular processes such as signal transduction, metabolism, cycle regulation, and apoptosis. YWHAZ is a potential regulator of β-catenin function, which is a central effector of Wnt signaling in tumorigenesis and metastasis. Exposure to tobacco smoke will lead to β-catenin in collaboration with interleukin-1β. Thus, YWHAZ could be a marker gene for pathological changes associated with tobacco smoke. Therefore, these two genes are used as the main target to be knocked down through gene therapy. It can reduce the risk of lung cancer (Cao et al., 2015).

3. Gene therapy

Gene therapy is a new treatment method that uses genetic material as a substitute for conventional medicinal compounds. This is to block the expression of any diseased gene or a new gene expressing a protein that is harmful to a cell. This is particularly suitable for treating infectious diseases and some cancers. The basic concept of gene therapy is to convert viruses or modify viruses into genetic carriers, which deliver the desired gene to target cells. Viruses that have been modified are used as vectors. In gene therapy, the DNA that codes for therapeutic

proteins is packaged in "vectors," which transport the DNA within the cells in the body. Because of its modification, it also can encounter extracellular and intracellular barriers must be non-toxic and non-immunogenic and must allow sufficient expression of the gene of interest. Gene therapy involves the introduction of one or more foreign genes into an organism (Patil et al., 2012).

Vectors for gene therapy can be classified into two types. they are viral vectors and non-viral vectors. Viral vectors used are Adenovirus, Retrovirus, Adeno-Associated Virus, Lentivirus, Vaccinia virus, and Herpes simplex virus; meanwhile, the non-viral vectors are Lipid complex, liposomes, Peptide/ protein, and Polymers (Patil et al., 2012).

4. Lentiviral

One method of gene therapy involves a virus as a vector. One of the viral vectors that can be used in gene therapy is lentiviral. Lentiviral vectors in gene therapy are methods that use lentiviral to insert beneficial genes into body cells, to modify genes, or to delete unwanted genes in organisms. Lentiviral is a virus having a viral envelope with prominent glycoproteins. It can help lentiviral to attach to the outer membrane of the host cell. In fact, many other viruses can be used as vectors in gene therapy, but lentiviral has a wider range of potential applications. This is due to the lentiviral uniqueness. If based on their nature, other retroviruses cannot penetrate the nuclear envelope. They can only act on cells that are undergoing mitosis. While the lentiviral uniqueness is having the ability to infect non-dividing cells. Thus, lentiviral can infect dividing cells or non-dividing cells. This is what makes the range of its potential applications wider (Cockrell2007).

Lentiviral in gene therapy are lentiviral that have been modified or manipulated, both gene manipulation and lentivirus structure. The manipulation is used to deliver certain genes to change the course of the disease. Part of the viral genome must be removed, so the virus can't replicate itself and can't be infectious. Lentiviral in gene therapy only have an envelope that will carry certain genes that are needed by the body's cells. It is replaced with a gene to be permanently inserted into the host cell genome using a previously genetically modified virus (Buchschacher, 2000).

5. shRNA

Short hairpin RNA or hairpin RNA (shRNA / Hairpin Vector) is an artificial RNA molecule with a tight hairpin loop that can be used to silence target gene expression. shRNA is a beneficial RNAi mediator because it has a relatively low rate of degradation and turnover (Wang, 2011). The introduction of shRNA into mammalian cells can use modified viral vectors, one of which is lentiviral (Paddison, 2002). After the vector is integrated into the host genome, shRNA is transcribed into the nucleus. Its expression is obtained from within mammalian cells, where the promoter part will become the binding site for RNA-polymerase III. the product is processed by Drosha. The resulting pre-shRNA is exported from the nucleus by Exportin 5. This product is then processed by Dicer. There is a deletion of the loop sequence leaving the stem sequence. Stem sequences are processed by the RNA-induced silencing complex (RISC). The sense strand is degraded. The antisense strand directs RISC to mRNA. It has a complementary sequence. RISC either cleaves the mRNA or represses translation of the mRNA. Thus, the shRNA leads to target gene silencing.

C. Methodology

1. Research Design

The offered method in this idea is based on reviews of related journals. It can find some procedures that can be applied to this idea. To prove this idea, it is necessary to conduct experimental laboratory research first.

2. Research Procedure

Based on this review, to test the effectiveness of shRNA, a research procedure must be carried out as follows. It includes making of shRNA design for CYP1A1 and YWHAZ (Each consists of a sense-loop-antisense), making of shRNA constructs on the vector lentivirus making, transducing of PAa adenocarcinoma cells, Western blot analysis, analyzing of cell cycle, and apoptotic cell (Jiang et al., 2017).

3. Technique of Data Analysis

Based on the literature, the promotor used for shRNA expression is amplified with forwards and reverses primers which are added by the appropriate restriction sites at both ends, and shCYP1A1 and shYWHAZ were also. The first shRNA-inserted plasmid (shCYP1A1) was restricted using a restriction enzyme corresponding to the shCYP1A1 end. The plasmid is then re-linearised using a restriction enzyme appropriate to the shYWHAZ end. It allows for subsequent ligation (shYWHAZ ligation). The fragment containing double cassette was re-restricted with an enzyme, so it just cuts the entire shRNA gene and its promoter. Meanwhile, to design the envelope vector, the Ebola virus glycoprotein sequences were obtained through PCR using forward and reverse primers for these genes that had been added with site restrictions. The glycoprotein sequence is ligated to the plasmid just downstream of the plasmid promoter (Spanevello, 2016). In addition, the viral packaging system construct was also designed. Each construct can be seen in Fig. 1, Fig. 2, and Fig. 3. This study must use three groups. They are no transduction, negative control (lentiviral transduction without shRNA), and target-shRNA (lentiviral transduction with shRNA). Three replications were carried out for each group (Jiang et al., 2017). After that, a western blot analysis was carried out. The analysis was repeated 3 replications. Quantitative analysis of CYP1A1 and YWHAZ protein expression was performed using ImageJ software. In addition, cell cycle and apoptotic analysis were also carried out by the FACScan ™ system and supported by the CellQuest ™ software (version 5.7; BD Biosciences).

D. Findings and Discussion

1. Findings

This literature study is expected to be a reference for researchers who are interested in the field of gene therapy. From this study, it is expected to be able to produce new treatment methods that use genetic material, as a substitute for conventional medicinal compounds. From this study, it was obtained the construct design as shown below.

Figure 1. Construct of Transfer Plasmid

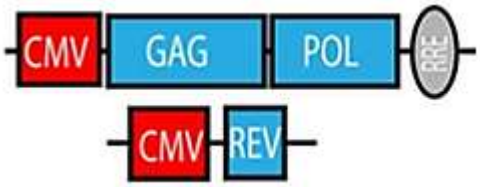

Figure 2. Construct of Packaging plasmid

Figure 3. Construct of envelope plasmid

This construct can be designed using the 3rd generation system accorded to addgene.org. The packaging system is split into two plasmids: one encoding Rev and one encoding Gag and Pol. In addition, Tat is eliminated from the 3rd generation system through the addition of a chimeric 5' LTR fused to a heterologous promoter on the transfer plasmid.

2. Discussion

The main cause of lung cancer is cigarette consumption which can send very high levels of nicotine to the brain and other organs such as the lungs (Barber et.al., 2008). The more you consume cigarettes, the more you are exposed to compounds that are substrates of CYP1A1 and YWHAZ genes. The genes will encode proteins playing a role in causing cancer, especially lung cancer. CYP1A1 is an enzyme coding gene that plays a role in catalyzing Polycyclic Aryl Hydrocarbons (PAHs), aromatic amines, and polychlorinated biphenyls into polar compounds. However, under certain conditions, this enzyme will catalyze the bioactivation of compounds that may react with macromolecules, which can cause the mutagenesis process (Barber et.al., 2008). According to Mohammed and Shervingto (2008), CYP1A1 is an extrahepatic enzyme

catalyzing the bioactivation of polyaromatic hydrocarbons (PAH) which are abundant in tobacco smoke. It can cause mutagens and carcinogens.

Meanwhile, the YWHAZ gene (also known as 14-3-3zeta), which encodes for the 14-3-3ζ protein, is located on chromosome 8q22.3, and is mostly found in breast NSCLC and other cancers (Fan, 2007; and Chen et al., 2012). YWHAZ has been identified as a clinically relevant prognostic marker for breast cancer, lung cancer, head and neck cancer, and hepatocellular carcinoma (Fan et al, 2007; Matta et al, 2008; Lu et al, 2009; Neal et al, 2009; Watanabe. et al., 2016). YWHAZ is a potential regulator of β-catenin function, which is a central effector of Wnt signaling in tumorigenesis and metastasis. Exposure to tobacco smoke will lead to β-catenin. It will collaborate with interleukin-1β. Thus, YWHAZ can be a marker gene in tobacco smoke-related pathological changes (Cao et al., 2015).

From this literature study, the process to produce these engineering virus particles is by transplanting three vector designs into A293T cells (Merten et al, 2016). Then, the engineered virus can be used to transduce the desired cells. Because the envelope vector used is glycoprotein originating from the Ebola virus, the envelope formed on the viral particles is ebolavirus glycoprotein. Chan et al., (2000) described that the application of pseudotyping vector lentivirus using ebolavirus and Marburgvirus glycoprotein has been successfully applied. These glycoproteins will facilitate the endocytosis of viral particles to target cells. The selection of lentivirus, like retroviridae as a vector, is based on the main advantage of lentiviruses. It has the ability to tranduce cells even in a non-dividing state (Escors and Breckpot, 2010; Buchschacher and Wong-Staal, 2018).

In addition, these particles also carry shRNA sequences because it is inserted by shRNA coding sequences for CYP1A1, and YWHAZ gene. The introduction of shRNA into mammalian cells by infection with viral vectors can make stable of shRNA integration. It also allows long-term knockdown of the targeted genes. When the virus particles are tranduced into PAa adenocarcinoma pulmonary cells, each gene will be transcribed to mRNA (Moore et al., 2010). The expressed hairpin mRNA will fold to the dsRNA. We know that as shRNA (hannonlab.cshl.edu, and origene.com).

The formed shRNA will be processed into siRNA. First, it is expressed in mammalian cells by introducing a plasmid vector. Its promoter part will be the binding site of RNA-polymerase III. This interaction is used to drive shRNA expression (Brummelkamp et al., 2002; Paddison et al., 2002; Sui et al., 2002; Yu et al., 2002; Abbas-Terki et al., 2002). ShRNA is one of the RNA interferences consisting of double-stranded RNA (dsRNA) which has a role in the degradation of specific mRNAs (Hayafune, 2006). Each shRNA will be transcribed separately because it contains a promoter and a terminator in each construct. Both will form a short hairpin structure. ShCYP1A1 and shYWHAZ will be processed by Drosha, then exported to the cytoplasm. They will be associated with Dicer. The loop sequence is deleted. The stem sequence is associated with RISC and one of the RNA strands is removed. The antisense strand directs RISC to mRNA. It has a complementary sequence. RISC either cleaves the mRNA or represses translation of the mRNA. Thus, the shRNA leads to target gene silencing. The schematic can be seen in Figure 4 (Fig. 4). shCYP1A1 will target complement sites of CYP1A1 mRNA, and shYWHAZ will target complement sites of YWHAZ mRNA.

Thus, there are 2 RNA interferences that work simultaneously in degrading mRNA having a role in causing lung cancer. This shRNA will inactivate the expression of target genes (McIntyre, and Fanning, 2006; Paddison et al., 2002). Hopefully, this therapeutic method will be effective in use.

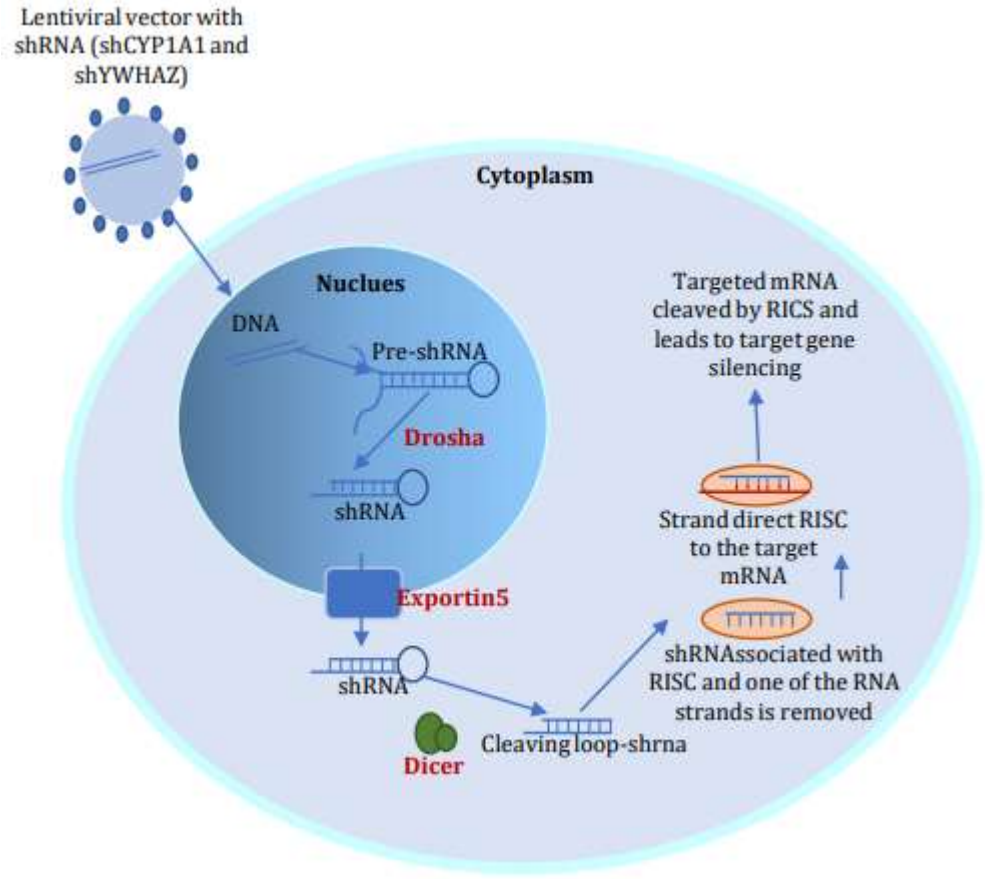

Figure 4. Schematic of in vivo targeted delivery of shRNA (adapted from O'Keefe, 2021)

When a western blot analysis was performed, it was expected that only a few or no CYP1A1 and YWHAZ protein bands were formed. Then, at the apoptosis analysis, it is expected that many cancer cells will experience apoptosis. This is due to the CYP1A1 and YWHAZ proteins unexpressed. The unexpressed CYP1A1 protein will not produce reactive metabolites involved in the initiation of carcinogenesis. Unexpressed YWHAZ protein will be no β-catenin regulation (no central effector of Wnt signaling, so there are no tumorigenesis and metastasis). Thus, nothing regulates the growth and differentiation rapidly. The oncogenic transformation isn't also occurs.

E. Conclusion

From this study, it can be concluded that the use of shRNA for CYP1A1 and YWHAZ gene therapy is able to block the formation of CYP1A1 and YWHAZ proteins. The chance of developing cancer can be minimized. Hopefully, the use of gene therapy is an alternative to reduce lung cancer cases.

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