Potential Treatment of Non-Small Cell Lung Cancer by Upregulating Zinc Finger Protein 671 Expression

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Abstract

Non-small cell lung cancer (NSCLC) is the most common type, with a high mortality rate. It is often diagnosed at an advanced stage, which causes treatment difficulties due to a lack of symptoms at early stages. Here, we propose a new approach for treating NSCLC via upregulating zinc finger protein 671 (ZNF671) expression. Firstly, we will reduce the degree of methylation at its gene promoter site in the NSCLC tumor cells. Secondly, the RT-qPCR technique is used to compare the expressions of ZNF671 between different methylation statuses; in vitro cell viability, proliferation, and invasion assays are applied to test the effects of demethylation in tumor cells. Finally, the patient-derived xenografts (PDX) model is used to observe the effect of regulating methylation status on the expression of ZNF671, affecting tumor growth in vivo.

1. Introduction

Lung cancer is regarded by WHO as the most common malignant tumors worldwide, and about 85% of all lung cancer cases are classified as non-small cell lung cancer (NSCLC) [1,2]. Among all NSCLC patients, a disturbing 75% of the total population are diagnosed at the advanced stage, indicating a dismal prognosis for this disease [3]. Furthermore, 90% NSCLC death cases are caused by exacerbation of cancer metastasis. Therefore, a treatment to target the metastatic ability of NSCLC cells shall be present, which will significantly increase the survival rate of NSCLC patients.

ZNF671 is a member of the Kruppel Associated Box Zinc Finger Proteins (KRAB-ZFP) family of mammalian transcriptional repressors, containing C2H2 zinc fingers and a Krüppel associated box (KRAB) domain. It is widely known for its function in inhibiting tumor cells migration and proliferation [2,4]. To validate ZNF671 as a biomarker for the diagnosis of lung cancer, a recent study has shown the overexpression of ZNF671 can restrain the proliferation and metastasis of lung cancer cells by blockage of Wnt/β-Catenin signaling pathway [2]. Some studies have also shown that the promotion of cancer linked hypermethylation in different types of cancer cells is directly associated with the downward regulation of ZNF671 [4]. Down-regulated ZNF671 in NSCLC patients is a cause of progressive cancer development and poor prognosis.

In this proposal, we describe a treatment method of NSCLC that focuses on the upregulation of ZNF671 transcription factor. Our hypothesis is that up-regulated ZNF671 will act as a tumor suppressor in NSCLC cell population, and perform its function by inhibiting the proliferation, migration and invasion of lung cancer cells. As one of the most fatal cancers worldwide, a treatment to advanced stage lung cancer is urgently required in order to increase the survival rate. This research aims to demonstrate the validity and possibility of treating advanced NSCLC by up-regulating ZNF671 in cancer cells. The possible positive result will validate its feasibility of our proposed method.

Our strategy to achieve the goal of promoting ZNF671 expression is by reducing the degree of promoter gene methylation. An overexpression of ZNF671 shall inhibit the proliferation, migration and invasion of tumor cells from the original site to another location, which can greatly alleviate the severity of the cancer. We will describe experimental methods to search for suitable demethylation molecules, later test the effectiveness and validity of treating NSCLC by reducing promoter methylation to upregulate ZNF671 expression [as shown in Figure 1].

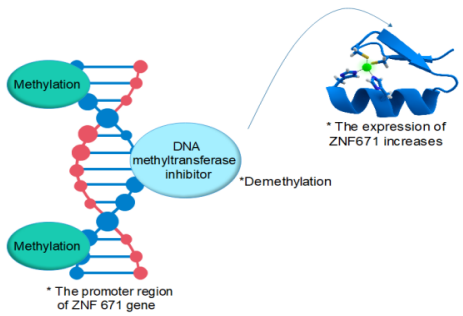

Figure 1 The process of reducing promoter methylation to upregulate ZNF671 expression

2. METHODS

2.1 Dataset Analysis

This paper used T-test to analyze the Data. The Gene sequence of ZNF671 and the major methylated promoter site are identified respectively from GDC Data Portal and GeneCards [5,6]. The target DNA methyltransferase inhibitors are obtained from Anticancer Research [7].

2.2 Immunofluorescence Analysis

Tumor tissue sample is collected from ten NSCLC patients at advanced stage by biopsy. For each patient, we divide the sample into 2 groups with equal quantities of cells. One is the control group and another is the experimental group. Then, the samples are washed by Phosphate-Buffer Saline (PBS) and fixed by 2% Paraformaldehyde solutions. After 15-minute fixation, the samples are washed by PBS three times for 10 minutes each. Next, 2% Bovine Serum Albumin is added to the samples to block the nonspecific binding.

Thirty minutes later, we added DNA Methyltransferase Inhibitor, Azacytidine, to the experimental group [as shown in Figure 2]. Then we apply the specific primary antibody targeting the hypermethylated CpG site at the promoter region of ZNF 671 gene, provided by the commercial immunofluorescence kit, to both groups so that it could bind to its epitope in PBS buffer [as shown in Figure 3]. After binding, the samples are incubated at room temperature for an hour. Next, the samples are washed again by PBS three times and each time takes 10 minutes. The secondary antibody provided in the kit is then added into the culture plate and binds to the primary antibody added before. After thirty-minute incubation, the samples are washed by PBS four times and each time takes 10 minutes. Next, 0.5 μg/ml DAPI is added to stain the secondary antibody and then the samples are washed by PBS three times and each takes 10 minutes. Finally, the samples are viewed by fluorescence microscopy to analyze whether Azacytidine has successfully bound to the hypermethylated CpG site, while at the same time verifying if tumor cells in our original sample have hypermethylated ZNF671 promoter region.

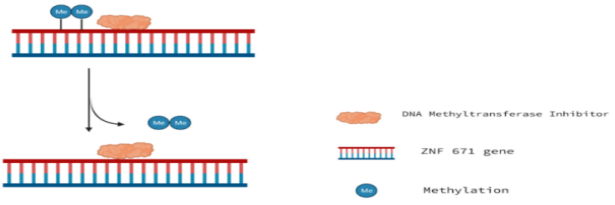

Figure 2 The expected process of DNA methyltransferase inhibitor binding to CPG site

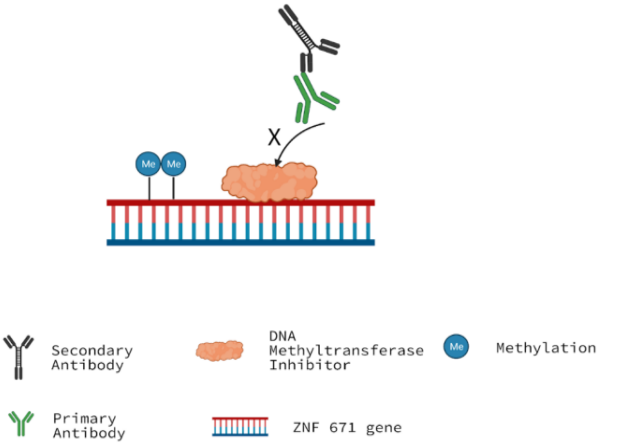

Figure 3 Mechanism of Immunofluorescence at CpG Site

2.3 Fluorescent probe-based RT-qPCR

In this procedure, we take the two samples processed in immunofluorescence analysis. The mRNA of ZNF 671 gene in both samples is extracted by a specific commercial mRNA Isolation Kit. We name the mRNA from the control group as RNA group one, and that experimental group as RNA group two. Then, both of the RNA groups are put into fluorescent probe-based RT-qPCR matching with their coupled primer. After running the process, a comparison of the quantification of mRNA in both of the groups can help analyze how the hypermethylated CpG site at promoter region influences the expression level of ZNF 671 [as shown in Figure 4].

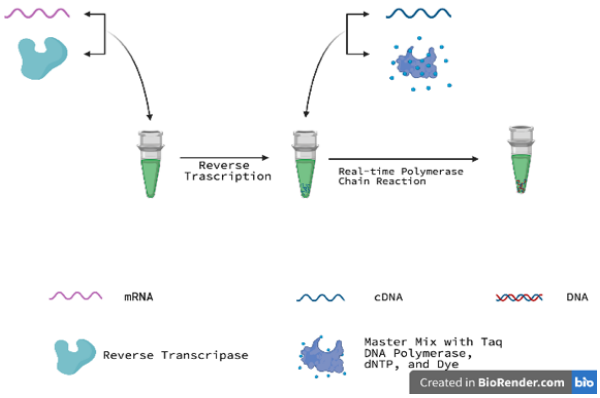

Figure 4 RT-qPCR for the expression of ZNF 671 gene

2.4 Cell Viability Assay

The cell viability of the tissue sample cells is tested using the MTT assay. Cells extracted from the target tissue sample are put into wells at a density of 2×103 cells/well. After treatment, culture with equal volumes of serum-free media and MTT solution. The plate is incubated for 4 hours in a 37 degrees Celsius environment followed

by addition of MTT solvent (dimethyl sulfoxide) to the wells. The plate is shaken for 15 minutes and a spectrophotometer is used to read the absorbance at 490 nm wavelength [2]. The MTT assay procedure is repeated after the cells are demethylated (adding DNA Methyltransferase Inhibitor, Azacytidine) in order to observe the cytotoxicity of the treatment.

2.5 Cell Proliferation and Invasion In Vitro Assay

Before demethylation of the ZNF671, proliferation assay is used to test its proliferation ability. Cancer cell samples are first extracted from the cancer tumor, mixed with 3-H thymidine (radioactive) in plate cells, and incubated for 24 hours. After incubation the cells are then run through a scintillation counter to detect cell proliferation by detecting newly synthesized DNA strands that incorporate 3-H thymidine. After tissue samples are demethylated (adding DNA Methyltransferase Inhibitor, Azacytidine), run through the same process of proliferation assay once again [8].

Lastly, a Boyden-chamber assay plate is used to evaluate the invasiveness of the cancer cells before and after demethylation. Plate wells are filled with FBS (Fetal Bovine Serum) medium. Cells in each well stay on top of a transwell insert membrane coated with Matrigel. The plate is incubated for 48 hours for invasive cells to cross and attach on the other side of the membrane. After 48 hours, the plate is then filled with a detachment buffer to detach the invasive cells off from the membrane. The membrane is then taken out of the well and CyQUANT GR Dyes are added to stain the invasive cells that are left. The number of cells is counted x200 magnification. The cell invasion assay process is then repeated after the cells of the target tissue sample are demethylated (adding DNA Methyltransferase Inhibitor, Azacytidine). The results from before and after the demethylation are contrasted.

2.6 Patient Derived Xenograft Animal Model

The tumor cells extracted from NSCLC patients are divided into two groups evenly; the control group contains no modification at ZNF 671 gene; the experimental group, the ZNF 671 gene is silenced [as shown in Figure 5]. Then, two groups of tumor cells are in situ injected into two groups of mice respectively [as shown in Figure 5]. All the mice are immunocompromised, and each group of mice are in the similar size, number, gender and age. Both of the groups are raised in the same environment and for three generations to make sure the tumor cells grow and graft in mice.

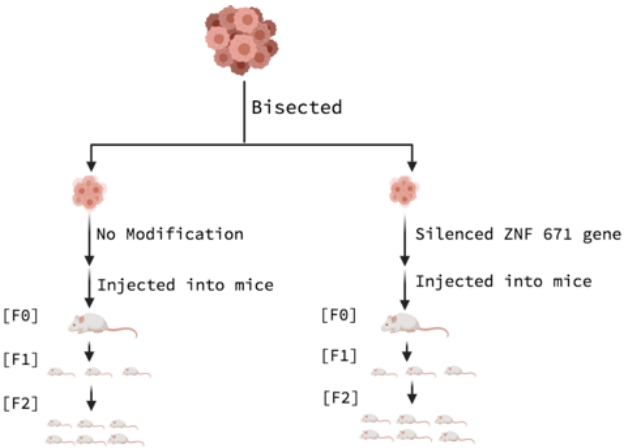

Figure 5 Patient Derived Xenograft Animal Model

One group of mice containing modified ZNF 671 gene is called the experimental group; another group of mice is called the control group. When the tumor cells grow at a constant rate in third generation of mice of the two groups, each group is divided into two sub-groups evenly, in which one sub-group of mice are injected with DNA methyltransferase inhibitors while the others are injected with the same volume of saline in the same way [as shown in Figure 5]. Finally, the number, size of tumor cells and the survival rate of tumor cells as well as the chance of finding tumor cells in other organs of the mice, in after several days, when the DNA methyltransferase inhibitor binds to target site, each sub-group are detected to analyze how different ZNF 671 genes and the DNA methyltransferase inhibitors affect the development of tumor cells.

3. RESULTS & DISCUSSION

3.1 In vitro experiment on the effect of demethylation of ZNF671 on its expression

Downregulation of ZNF671 has been associated with enhanced cell proliferation and tumorigenicity in various cancer types, including nasopharyngeal carcinoma and NSCLC that we will research on [2,8]. Hypermethylation of ZNF671 is implied as a direct cause in NSCLC development, so we primarily want to study the therapeutic effects as we reduce the level of methylation of ZNF671 promoter gene [2]. One of the two pre-prepared NSCLC tumor tissue sample groups is set as the control group, while the other is added with artificially synthesized DNA methyltransferase (DNMT) inhibitor Azacytidine. We attach a coupled set of primary and secondary antibodies from a commercial immunofluorescence kit, in which the primary antibody specifically binds to the methylated CpG site at the

ZNF671 promoter gene. Through staining the secondary antibody with DAPI, the cases of successful antibody binding can be distinguished under the fluorescent microscopy. If we find a less intense fluorescence in our experimental sample, it verifies that the Azacytidine molecules have successfully bound to the CpG sites at the promoter regions of ZNF671 [as shown in Figure 6].

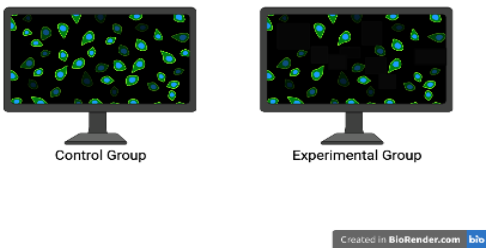

Figure 6 The expected fluorescence microscopy result when Azacytidine binds to the hypermethylated promoter region of ZNF671

With these results in hand, we will quantitatively compare the expression of ZNF671 between the control and experimental groups by measuring its mRNA quantity through RT-qPCR. If there is a statistically significant difference between the PCR results, we could imply that reducing the methylation of ZNF671 promoter gene affects its expression. Otherwise, this method does not directly influence the expression of ZNF671, or it needs to cooperate with other factors or cellular mechanisms overlooked in this proposal.

3.2 In vitro test results at the cellular level: the effects of demethylation treatment on tumor cell viability

By comparing the changes in cell viability before and after demethylation of non-small cell lung cancer tumor sample cells, it can be concluded whether the treatment factor is toxic to tumor cells or has other effects on cell viability. After the MTT test, the possible results can be divided into three types: the cell viability of the sample becomes larger, almost unchanged, and decreases. If the cell activity becomes larger, it indicates that the treatment factor will make the tumor cells grow better, which is not conducive to the treatment of the corresponding disease. If the cell activity is almost unchanged, then the treatment factor does not affect the viability of tumor cells. It is expected that the inhibitor can reduce the viability of tumor cells, but this does not necessarily mean that the growth and migration of tumor cells will be inhibited.

Therefore, from the perspective of this test, we cannot predict the therapeutic effect of demethylation. We need to analyze the effect of this treatment on tumor cells combining with other functional assay results. If the cell viability decreases which is our expected result, demethylation treatment reduces the activity of non-small cell lung cancer tumor cells. It is preliminarily confirmed that at the cellular level, this treatment factor has a certain effect on inhibiting the viability of tumor cells.

3.3 In vitro test results at the cellular level: the effects of demethylation treatment on tumor cell proliferation, migration and invasion ability

The 3-H thymidine-labeled proliferation assay was used to detect the proliferation ability of tumor cells and the Boyden-chamber assay was used to detect the migration and invasion ability of tumor cells. By comparing the cell state before and after the demethylation treatment through this experiment, several possible results can be obtained: the cell proliferation ability is reduced, unchanged, and enlarged, and the cell migration and invasion ability is reduced, unchanged, and enlarged.

Among them, the cell proliferation, migration and invasion ability decrease at the same time, which is the most promising result we think. This indicates that demethylation therapy demonstrates its inhibitory effects on tumor cell proliferation, migration and invasion at the cellular level simultaneously, which is of great significance for the treatment of non-small cell lung cancer.

3.4 In vivo experiment: the effect of demethylation on tumor cell development

In vivo experiments can usually be closer to the real situation of life, so the reliability of the experimental results is higher and more valuable. This experiment uses mice as experimental objects. We use humanized immunodeficient mice that were in situ injected with the patient's non-small cell lung cancer cells for experimental observation and detection. PDX can better simulate the growth of patient tumors, and can reproduce the heterogeneity of the primary tumor, and is widely used in the screening of anti-tumor drugs.

Before and after treating with demethylation drugs, we will count and compare the number and size of tumor clusters in these four groups of mouse models to detect its proliferation and migration ability, and record the survival rate for statistics, and also compare the number of tumor clusters and autopsy to detect if cancer cells are found in other organs to detect its metastatic ability. Dividing all the results into three groups for comparison, namely group one and group two, group two and group four, as well as group one and group three. We can draw conclusions.

Table 1 Possible results for four different treatment groups

The impact on the tumor cells of mice after different treatments	Injecting saline	Injecting DNA Methyltransferase Inhibitor
ZNF 671 gene is silenced	*Group one:* The number of tumors Tumor size Survival rate of cancer cells The chance of finding cancer cells in other organs of the mice	*Group three:* The number of tumors Tumor size Survival rate of cancer cells The chance of finding cancer cells in other organs of the mice
No modification	*Group two: control group* The number of tumors Tumor size Survival rate of cancer cells The chance of finding cancer cells in other organs of the mice	*Group four:* The number of tumors Tumor size Survival rate of cancer cells The chance of finding cancer cells in other organs of the mice

***** There are three possible results for each parameter measurement. Significantly increase, significantly decrease and no significantly changes compared with the control group.

Comparing group one and group two [as shown in Table 1]: the comparison of these two sub-groups can intuitively show whether the expression of ZNF671 affects the viability, proliferation and metastasis of the cancer cells. Among the results, in group two, the number and size of cancer cells are smaller, and survival rate as well as the chance of finding tumor cells in other organs is lower, which is the result we hope to obtain. It shows that the higher expression of ZNF671 does inhibit tumor viability, proliferation and metastasis. Therefore, it is a good direction for us to find a way to upregulate the expression of ZNF671.

The comparison between group two and group four [as shown in Table 1] in the same aspects is to verify the effect of the inhibitor on tumor viability, proliferation and metastasis by regulating the expression of ZNF671.

The comparison between group one and group three [as shown in Table 1] is to exclude other effects of inhibitors on tumor cells.

Similarly, the results which are the most promising possible is that the result of group one and group three are roughly the same. At the same time, the result of group four decreases compared with the control group. These results indicate that the injection of DNA methyltransferase inhibitor in the mouse model can effectively inhibit the viability, proliferation and metastasis of tumor cells by regulating the expression of ZNF671, and the inhibitor will not affect their viability, proliferation and metastasis in the cells through other channels. It proves that the demethylation of the ZNF671 gene can be used as a treatment.

4. CONCLUSION

In this study, we test before and after the DNA methyltransferase inhibitor's effect: whether the inhibitor can bind to the CPG site of the promoter region of ZNF671 gene, the expression of ZNF671, tumor cell viability, proliferation, migration and invasion, as well as the growth changes of tumor cells in PDX. Through these experiments step by step from in vitro to in vivo, we verify the feasibility and effectiveness of demethylation treatment.

Our research provides a new trial method for the treatment of NSCLC. DNA methylation will be developed from a prognostic factor to a therapeutic target in the treatment of NSCLC, as the upregulation of ZNF671 could contribute to treating non-small cell lung cancer via inhibition of tumor cell proliferation, migration and invasion [9,10]. Further applications of the hyper-methylation downregulation process may be applied to other zinc finger proteins as well as on other cancers.

When this study is conducted, the main process is that the DNA methyltransferase inhibitor binds to the the CpG site, at ZNF671 gene promoter regions, thereby demethylating the ZNF671 gene. Subsequently, the expression of ZNF671 is regulated, which affects the proliferation, migration and invasion of non-small cell lung cancer cells. However, In fact, we are not sure whether the DNA Methyltransferase(DNMT) Inhibitors , Azacytidine can bind to the CpG site at ZNF671 promoter regions well. Only when the binding is successful can we proceed to the next step of experimental verification. The key to this proposal is identifying a suitable inhibitor which is crucial

to our verifying the demethylation treatment.

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Rui Liang and Sicong Shan are the co-first authors of this paper

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