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Identification of Target Proteins for Promoting Nuclear Envelope Rupture in Cancer Cells

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ABSTRACT

The nuclear envelope separates and protects DNA from the cytoplasm. In cancer cells, frequent nuclear morphological abnormalities and transient rupture of the nuclear membrane promote DNA damage and cancer malignancy. However, nuclear envelope instability, acting as a tumor-promotion factor, can be viewed as a fatal weakness of cancer cells. Although the exact mechanism is not clear, it is known that the loss of the tumor suppressor gene TP53 is involved in nuclear envelope instability in cancer cells. In this study, we demonstrate that the inhibition of NUP93, which is a subunit of the nuclear pore complex, induces cell death by enhancing nuclear envelope instability. The RNA interference (RNAi) inhibiting NUP93 expression in the human cell line RPE1 caused nuclear envelope rupture, and DNA damage, leading to cell death. Under the condition in which the tumor suppressor gene TP53 was simultaneously suppressed with NUP93, nuclear envelope rupture and DNA damage were significantly increased. Conversely, cell death was slightly decreased. The decrease in cell death may be ascribed to the fact that TP53 is involved in the induction of apoptosis by DNA damage. We speculate that amplification of nuclear envelope instability may induce cancer cell-specific synthetic lethality in the context of TP53-independent nuclear envelope instability. Moreover, our results suggest that nuclear pore proteins can be promising targets for the development of synthetic lethal anticancer drugs

Research Goals and Purpose

Research Purpose

Substances that can be used as anticancer agents should induce the death of cancer cells while not exhibiting severe toxicity in normal cells. The cellular specificity of anticancer drug action is an important task. Recently, using synthetic lethality as a strategy to increase cancer cell specificity is attracting attention [1]. Synthetic lethality is a concept in oncology that describes a phenomenon in which a malfunction in one of several specific genes does not affect the survival of a cell or organism, but a problem in the function of two genes induces cell death [2]. A drug that inhibits the function of a gene that works in pair with a gene that has frequent defection in cancer cells is likely to have a synthetic lethal effect. In normal cells, even though the function of a specific gene is suppressed, apoptosis is not induced because the alternative genes can partially substitute its role. For the development of synthetic lethal drugs, it is important to discover genes that work in pairs.

One of the biggest characteristics of cancer cells is that their cell proliferation control system is damaged. Due to the malfunction, unlike normal cells, cancer cells divide continuously. In addition, the homeostasis disturbance of cell nucleus size and shape can be considered a general feature of cancer cells [3]. In cytopathology, nuclear morphology is used as an important criterion for diagnosing cancer. Recently, it has been discovered that cancer cell nuclei are not only morphologically abnormal but also have poor membrane stability [4]. In particular, the repeated destruction and regeneration of the cancer cell's nuclear membrane were observed [5]. Since the nuclear membrane is a structure that protects DNA, which is necessary for cell survival, lethal damage to its structure induces cell death [6]. In normal cells, various nuclear membrane stability protection mechanisms work, but in cancer cells, membrane instability is high due to the

abnormal function of nuclear membrane stability maintenance genes. Therefore, drugs that promote nuclear membrane instability are expected to induce stronger apoptotic effects in cancer cells than in normal cells. Not much research has been conducted on whether cancer cell nuclear membrane instability can be a point of action for synthetic lethal drugs.

Research Goal

In previous studies, it was observed that when the expression of proteins which constitute nuclear pores in the nuclear membrane was suppressed, the shape of the cell nucleus was distorted, and the rate of cell death increased. This result shows the possibility that the stability of the cell nucleus can be suppressed by regulating the expression of the nuclear pore protein. The study aimed to clarify that the inhibition of the nuclear pore protein NUP93 can exhibit a synthetic lethal effect by amplifying cancer cell-specific nuclear membrane instability.

Theoretical Backgrounds

Nuclear Pore

A nuclear pore is a hole on the surface of a nuclear envelope and undergoes selective exchange of molecules between the cell nucleus and cytoplasm. It is composed of various proteins that form a molecular passage. Light substances with a mass of less than 60 kDa pass by passive transport, and heavy substances such as DNA and RNA pass by active transport [7,8]. Nuclear pores are made with a specific structure called a nuclear pore complex (NPC), and each NPC is composed of 400 or more proteins. Every cell has thousands of NPCs and each has a diameter of around 120 nm [9]. NUP93 protein, or nucleoporin 93, is essential for the composition and function of the NPC [10,11].

TP53 and NUP93 Gene

TP53 is a well-known gene for repressing tumors. It stops the cell cycle or induces apoptosis if the cell's genome malfunctions. The TP53 gene of cancer cells is depressed in a high probability because of gene defection or mutation. Additionally, it was reported that the nuclear envelope is ruptured 8~9 times during one cell cycle in cancer cells whose TP53 gene is deficient [12]. NUP93 is a gene that encodes nucleoporin 93. Nucleoporins are proteins that compose nuclear pores. Also, they are essential to maintain the nuclear structure of the human cell[13]. In this study, the instability of the nuclear envelope in cancer cells was imitated by repressing the TP53 gene using RNA interference. Also, the experiment aimed to show that repression of the NUP93 gene can induce amplification of nuclear instability and cancer cell death when the nuclei are destabilized by TP53 control.

RNA Interference

RNA interference is a phenomenon in which RNA is specifically repressed by nucleic sequences. Generally, double-stranded RNA such as micro-RNA (miRNA) or short-interference RNA (siRNA) causes RNA interference. Through this mechanism, organisms can control gene expression. Usually, RNA interference is undergone by miRNAs which are RNAs that are not encoding data for protein synthesis. Initially, micro-RNA is a very long nucleotide but is shorted to about 22 nucleotides when fully processed. miRNA decomposes messenger RNA with complementary nucleotides or interrupts translation in ribosomes, suppressing gene expression [14,15]. RNA interference is widely used as a tool to inhibit gene expression. Especially, siRNA, which is a synthetic nucleotide of about 19 to 24 nucleotides, is widely used because it functions similarly to miRNA in

vivo. siRNA suppresses gene expression by decomposing mRNA or interrupting translation [16].

NLS-EGFP and 53BP1-mApple

In this study, NLS-EGFP was used as a reporter protein to visualize nuclear envelope rupture, and 53BP1-mApple was used as a reporter protein to check DNA damage. NLS-EGFP is composed of a nuclear localization signal (NLS) and enhanced green fluorescent protein (EGFP). NLS is a common marker for proteins transported into the nuclei [17]. Proteins with NLS amino acid sequences strongly bind to importin, a transport protein, and move into the nuclei through nuclear pores [18]. The Ran-GTP protein binds to the importin-protein complex and weakens the affinity between importin and protein. Then, the protein is released from the importin, which is inside nuclei. After release, the Ran-GTP protein and importin complex move out of the cell nucleus through nuclear pores. Importin inside the cytoplasm delivers NLS combined with EGFP protein into the nucleus again. This process accumulates NLS-EGFP inside the nucleus of the cell. When the nucleus ruptures, NLS-EGFP leaks out temporarily. The leaked protein can be detected and quantified by a fluorescence microscope. Thus, NLS-EGFP can be used as a signal of nuclear rupture. 53BP1-mApple is a protein that is a complex of 53BP1 protein and fluorescent marker mApple. The 53BP1 protein is a protein that participates in intracellular DNA damage repair; it specifically repairs DNA double helix breaks [19]. The 53BP1-mApple reporter protein accumulates at the DNA breakage site and emits bright red fluorescence. Therefore, a fluorescence microscope can confirm DNA damage by observing 53BP1-mApple reporter protein foci (small dot-like structures that are brighter and more vivid than the surroundings).

Experiment Methods

Experiment Design

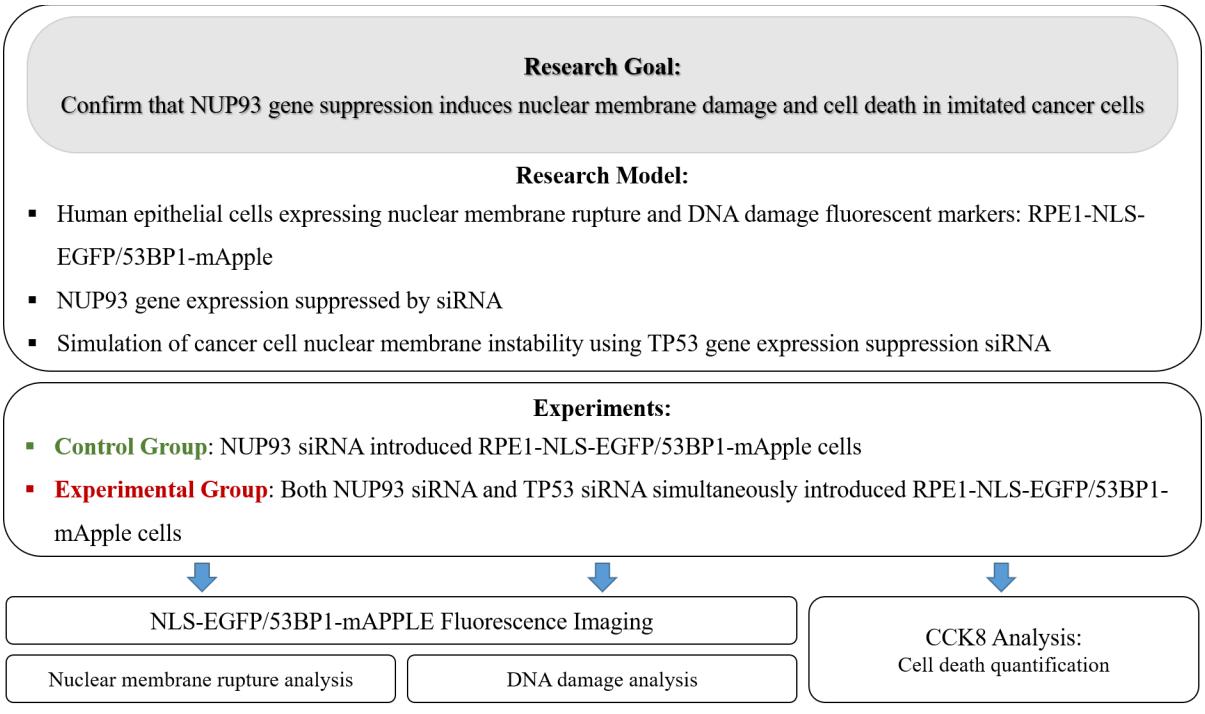

Figure 1. Research Flow Chart

The experiment aims to prove that the control of the NUP93 gene can specifically induce the disorder of the nuclear envelope and the apoptosis of cancer cells.

Cell Line

The cell line used was human RPE1-NLS-EGFP/53BP1-mApple. NUP93 siRNA exposed cells were the control group, and NUP93 siRNA-p53 siRNA exposed cells were the experimental group.

Experiment Equipment

For the culture of the human retinal pigmented epithelial cell (RPE1 cell), Dulbecco's Modified Eagle medium was used as media. 10% FBS 50U/mL penicillin and 50μg/mL streptomycin solution also have added to the media. Streptomycin solution also was added to the media. 0.25% trypsin and 1mM EDTA added Phosphate-Buffered Saline (PBS) are used for subculture. Cell line for fluorescence imaging is cultured on an 8-well Lab-Tek slide. Cells were fixed under 4% paraformaldehyde solution for 10 minutes and stained by a mounting solution containing 1μg/mL DAPI dye and 50% Glycerol. Lipofectamine RNAiMAX, a lipofectamine transfection solution, was used to inject siRNA into the cell.

Transfection of siRNA

Before the experiment, siRNA, lipofectamine, and OptiMEM were kept in ice, and five e-tubes and solutions (siRNA, lipofectamine, and OptiMEM) were prepared as an amount of [Table 1, 2]. The consistency of siRNA solution targeting TP53(siTP53) is 10nM, and other siRNA solutions are 20nM. For convenience, all four solutions, including OptiMEM, will be denoted solution A. The compositions of all four solutions are shown in [Table 1].

Table 1. Composition of solution A

e-tube # / siRNA type	e-tube 1	e-tube 2	e-tube 3	e-tube 4
siCONTROL	0.45μl	0.15μl	0.3μl	-
siNUP93	-	0.3μl	-	0.3μl
siTP53	-	-	0.15μl	0.15μl
OptiMEM	50μl	50μl	50μl	50μl

The amount of siCONTROL is modulated to equalize the total amount of siRNA solutions. Solution B is a mixture with the proportion shown in [Table 2].

Table 2. Composition of solution B

Reagent	Amount of Reagent
OptiMEM	225μL
Lipofectamine-RNAiMAX	5.4μL

50 µL of solution B was added to each e-tubes with solution A and reacted at room temperature for 25 minutes. While solution A and solution B were reacting, to prepare cells to spread in an 8-well Lab-Tek, the cells were subcultured. 1.2 mL of media and an appropriate number of cells were added to a 5 mL microtube and pipetted to make 30% cell confluency. After solutions A and B were incubated, each 50 µL of solutions in e-tube 1 to 4 were added to a separate well in an 8-well Lab-Tek. 250 µL of cell solution was added to each well and incubated in an incubator for 48 hours.

Fluorescence Imaging Using a Fluorescence Microscope

Incubated cells were observed by a fluorescence microscope (Deltavision Imaging System). The microscope made an image of cells and the program ImageJ divided the image into three RGB layers. In the case of the 53BP1-mApple fluorescence image, the number of cells with three or more red dots, which are cells with a disorder in their DNA, were counted. Likewise, in the NLS-EGFP fluorescence image, the number of cells whose green fluorescence was leaked out from the nucleus, which are likely to be nuclear envelope-disordered cells, were quantified.

Cell Death Analysis

After 72 hours of the addition of siRNA, the cell death rate of the cells was analyzed using the Cell Counting Kit 8(CCK8). After cell culture, the medium in each well of the 96-well plate was eliminated by the ASPIRATION 96 Program. 10 µL of CCK8 solution was added to each cell well. Cells were incubated for 2 hours in a 37℃ incubator. The 450nm light absorbance rate was recorded. By subtracting the absorbance rate of the well, which contains control cells, the death rate of cells was evaluated. Absorbance was measured using a spectrometer.

Results

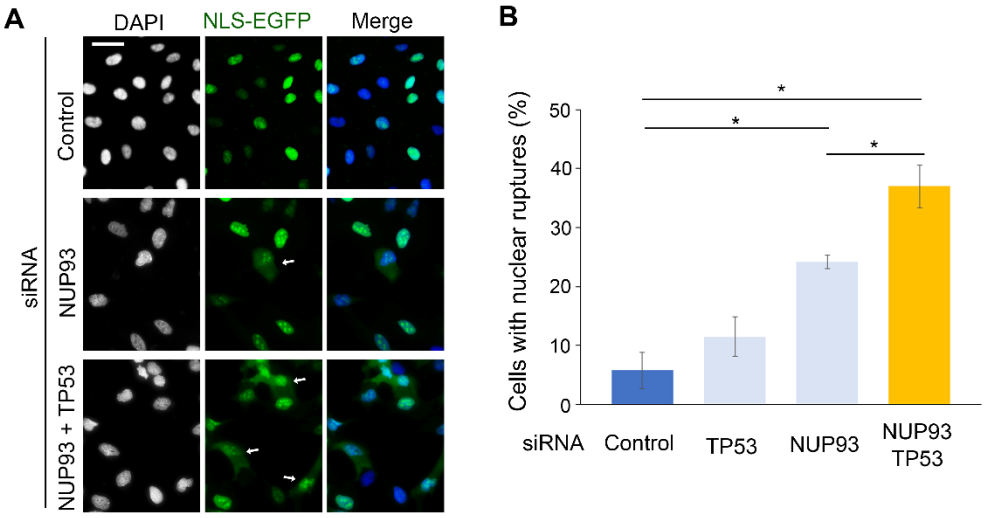

Figure 2. Effect of NUP93 and TP53 Knockdown on Nuclear Envelope Integrity Evaluated by NLS-EGFP Fluorescence. **2A**. NLS-EGFP image of NUP93 or TP53 knockdown on RPE1 cell line. **2B**. Ratio of cells whose envelope was ruptured when NUP93 and P53 genes are suppressed. (n=3, Error bar represents standard error of the mean)

NLS-EGFP reporter protein is contained in cell nuclei. Thus, NLS-EGFP was used to visualize the rupture of the nuclear envelope. The cells whose NLS-EGFP fluorescence was leaked out of the cell nucleus were considered to be nucleus-ruptured cells for each fluorescence image. (Refer to the white arrows in Fig. 2) Nuclei were identified by DAPI fluorescence which combines with DNA in the cell. The experiment compared four groups (control, TP53 knockdown, NUP93 knockdown, and NUP93+TP53 double knockdown group) and was run three times independently. The difference in the number of ruptured cells was evident between the four groups at a 95% significance level. (ANOVA test) The ratio of the ruptured cells was largest in the double knockdown group, followed by the NUP93 knockdown group, TP93 knockdown group, and control group. The result shows that NUP93 is essential to maintain the nuclear envelope stable. Also, the result shows that minor instability caused by TP53 knockdown was significantly amplified by NUP93 control. As in Fig. 2A, the cells of the control group usually had an elliptic cell envelope, while a high proportion of cells in the double knockdown group had dented envelopes.

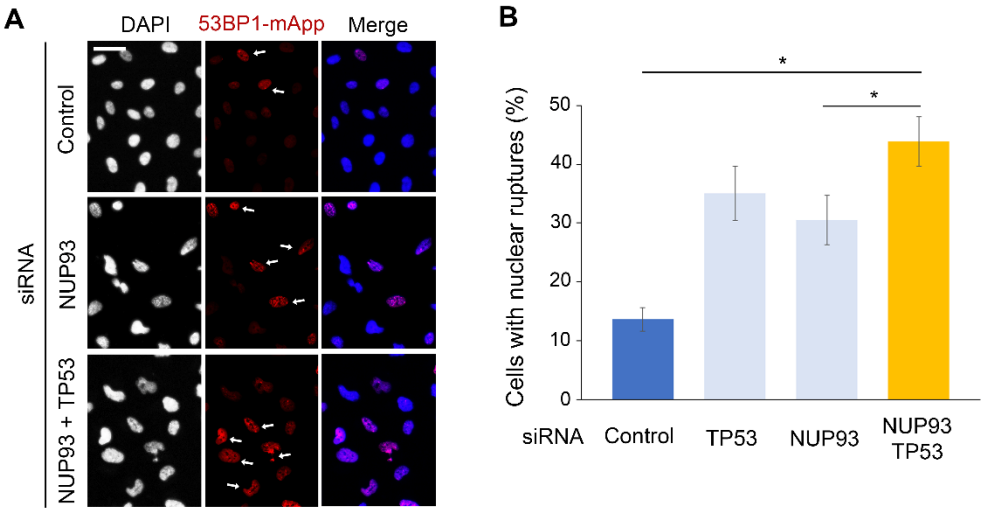

Figure 3. Effect of NUP93 and TP53 Knockdown on DNA Integrity Evaluated by 53BP1-mAPPLE Fluorescence. **3A**. 53BP1-mApple image of NUP93 or TP53 knockdown RPE1 cell. **3B**. Ratio of ruptured cell when NUP93 and P53 genes are suppressed. (n=3, Error bar represents standard error of the mean)

As in Fig. 3, some cells whose TP53 or NUP93 gene had been controlled showed red fluorescence from 53BP1-mApple protein, which implies DNA damage. (Refer to the white arrows in Fig. 3) The difference in the ratio of the damaged cells was significant among groups at a 95% significance level (p-value < 0.05) along three independent runs of the experiment in the same order for the evaluation of nuclear envelope rupture. (Ratio: Double knockdown > NUP93 knockdown > TP53 knockdown > Control) With the increased fluorescence density of 53BP1-mApple in the nuclei, the ratio of cells with red dot shape foci, which are found in DNA-damaged cells, also appeared significantly high in the double knockdown group. This result coincides with the previous experiment and can be interpreted that the double knockdown of NUP93 and TP53 genes effectively induces envelope rupture. More than around 10% of cells in the control group showed clear 53BP1-mApple fluorescence. These cells are presumed to show DNA damage and repair processes during DNA replication. As an additional notable feature, cells with micronuclei with strong fluorescence were found in the double knockdown group.

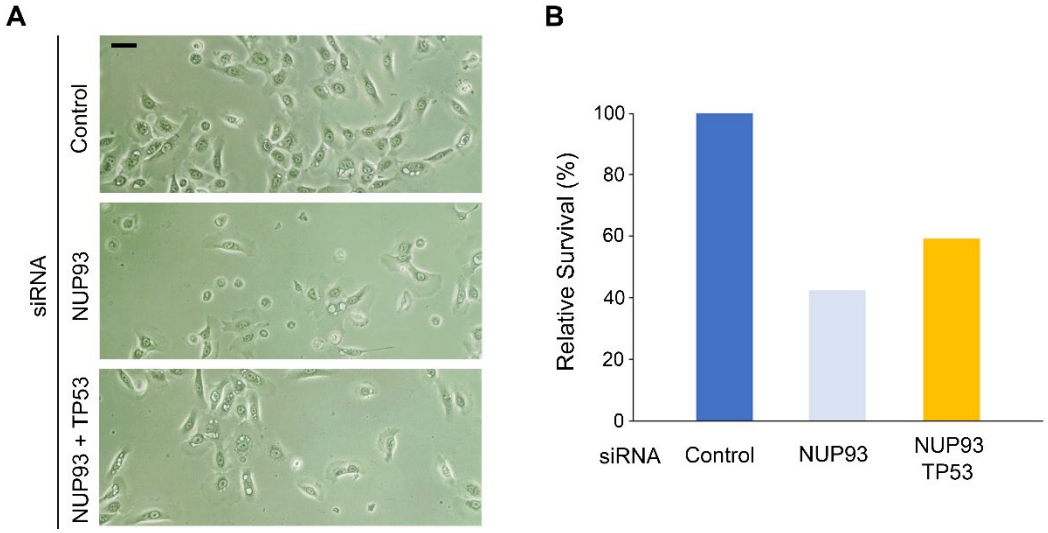

Figure 4. Quantification of Cell Survival After NUP93 and TP53 Knockdown Using CCK8 Assay. **4A**. Image of NUP93 or TP53 knockdown RPE1 cell. **4B**. Ratio of survived cell when NUP93 and P53 genes are suppressed.

CCK8 analysis was conducted to verify that nuclear membrane rupture and DNA damage can be a direct cause of cell death. This study compared the control group, NUP93 knockdown group, and NUP93+TP53 double knockdown group once. The data of the control group was used as the standard for comparison. The experiment was not repeated for biological replicates. Thus, error bars were not marked on the figure and statistical significance was not analyzed. In NUP93 knockdown group, cell death increased by more than 50%. Contrary to expectation, fewer cells died in the double knockdown group than in the NUP93 knockdown group. This phenomenon is thought to be because the TP53 gene is related to the apoptosis of DNA-damaged cells. Therefore, cell death of DNA-damaged cells may decrease when TP53 is inactivated. In Fig. 4A, the bubble-shaped structure is found in all three groups. It appears to be the product of a lipofectamine reaction and is likely not to be the effect of the knockdown of a specific DNA. Also, the cells' density was less in the NUP93 knockdown group and double knockdown group than in the control group. This can be another evidence that the cell dies when the NUP93 or TP53 gene is inactivated.

Discussion

Nuclear membrane instability and DNA damage were analyzed using reporter proteins NLS-EGFP and 53BP1-mApple. Under the TP53 suppression condition, when the expression of the NUP93 protein, a nuclear pore component, was suppressed, the frequency of nuclear morphological transformation and nuclear membrane rupture significantly increased. In addition, a high level of DNA damage was observed in the cell group in which both the TP53 gene and the NUP93 gene were inactivated. Consequently, a significant correlation was found between nuclear membrane rupture, DNA damage, and cell death. The fact that nuclear membrane instability increased in cells in which both TP53 and NUP93 genes were inactivated compared to the control or in which only one of the TP53 and NUP93 genes were inactivated indicates that NUP93 is a valuable anticancer drug target.

TP53 is a tumor suppressor gene and often malfunctions in a cancer cell. The study used a model in which the expression of TP53 is inactivated by siRNA. Using the model, the nuclear membrane instability of cancer cells was simulated. As shown in Fig.2 and 3, an increase in nuclear membrane rupture and DNA damage was observed. Furthermore, nuclear membrane instability amplification appeared when NUP93 inhibition was applied. However, in cell death quantification experiments, the cell death effect by NUP93 siRNA was higher in cells with normal TP53 function compared to cells whose TP53 was suppressed. This phenomenon may be because of the function of TP53. When TP53 is

normal, nuclear membrane rupture and DNA damage caused by inhibition of NUP93 expression can induce apoptosis through the TP53 pathway activation. However, when TP53 is malfunctioning, the pathway may not work. Therefore, the simultaneous inhibition of the TP53 and NUP93 genes can reduce cell death. The results show that suppressing the TP53 gene to simulate nuclear membrane instability in cancer cells was not optimal for analyzing the synthetic lethal effect. Also, the high rate of cell death NUP93 suppression showed in CCK8 assays suggests that the inhibition of NUP93 in normal cells may show significant toxicity. Thus, further studies are required to confirm that the NUP93 gene is an optimal drug target. Additionally, a model that suppresses NUP93 with a tumor suppressor gene that is not involved in the apoptosis pathway would be helpful.

Cancers occur in various organs and tissues of the human body. Each has different sensitivities to anticancer drugs depending on the site and type of occurrence. Therefore, studies will be needed to select carcinomas most responsive to NUP93-targeting drugs. Experiments to compare and analyze the cytotoxicity of NUP93 inhibition in normal cells of each body part are also required. The experiment confirmed that nuclear membrane rupture and cell death occurred when siNUP93 was introduced. However, the phenomenon might have originated from a non-specific (off-target) effect of siRNA. Therefore, an additional experiment (such as a rescue experiment) is needed to observe whether the knockdown traits will be improved by introducing a NUP93 expression vector, which is not affected by siRNA, into the cell.

Many researchers are putting effort into developing anticancer drugs. However, according to CDC, the number of new cancer patients in the US continues to increase, with 1.6 billion in 2009, 1.7 billion in 2014, and 1.8 billion in 2019. In 2015, the medical care and oral drug cost for cancer patients was 182.65 billion dollars only in the United States [20]. The social cost will increase if death losses due to early death are added. Therefore, developing better anticancer drugs is an important social task. In this study, a new drug target was researched to establish the basis for developing a new mechanism of an anticancer drug. As shown in the study, nuclear pore proteins, especially NUP93, has the potential to be used as drug targets for cancer treatment. Although it is not certain that NUP93 is the optimal drug target, it is expected that follow-up studies will open the way for developing innovative synthetic lethal anticancer drugs that use the nuclear pore as the action point.

Conclusion

The control, TP53 suppression, NUP93 expression suppression, and TP53-NUP93 double suppression groups were compared in this study. As a result, the frequency of nuclear envelope rupture increased in the double suppression group compared to the TP53 and NUP93 suppression groups alone. Also, DNA damage, which was indirectly confirmed by the reporter protein, was found to be significantly higher in the double inhibition compared to the NUP93 and TP53 expression inhibition groups. A higher cell survival rate was observed in the double expression inhibition group than in the NUP93 inactivation group.

According to these results, cell envelope rupture and DNA damage can be induced by NUP93 gene inhibition suppression in cancer cells whose TP93 gene is commonly malfunctioning. In addition, if the function of NUP93 is completely inhibited in normal cells, a high level of cytotoxicity is expected to appear. Therefore, further experiments will be required to confirm whether NUP93 inhibition will have a synthetic lethal effect on cancer cells.

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