Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Inhibition mechanism of PinX1 Gene on cancer stem cells of nasopharyngeal carcinoma

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ARTICLE INFO

Original paper

Article history:
Received: July 14, 2022
Accepted: September 27, 2022
Published: September 30, 2022

Keywords:

nasopharyngeal carcinoma, pinx1 gene, tumor stem cells, telomerase activity

ABSTRACT

The recurrence and long-term metastasis of these tumors are important causes of treatment failure and death. On the other hand, PinX1 is a nucleolar protein found in recent years that can interact with telomere/telomerase simultaneously, and it is highly conserved in human and yeast. Some studies have shown that the PinX1 gene can inhibit the tumor stem cells of NPC. Therefore, the mechanism of inhibition of the PinX1 gene on the tumor stem cells of NPC has been studied in this paper. In this paper, CNE2 cells of NPC were used as experimental materials, CD133 as a marker, PinX1 overexpression plasmids and their corresponding empty plasmids were respectively transfected in CD133+ cells, PinX1 siRNA and their corresponding NC siRNA were respectively transfected in CD133- cells for control experiments. In this study, we found that the telomerase activity of the CD133 - + NC group was 1.001 ± 0.086, the CD133 - + pinx1sirna group was 0.974 ± 0.046, CD133+ + vector group was 0.928 ± 0.102, CD133+ + over PinX1 group was 0.703 ± 0.086. Therefore, the PinX1 gene can inhibit NPC stem cells by inhibiting telomerase activity.

Doi: http://dx.doi.org/10.14715/cmb/2022.68.10.28

Introduction

Compared with other tumors, nasopharyngeal tumors have the characteristics of fast growth and low differentiation of cancer cells, and they are more sensitive to radiation. Moreover, the structure of the disease site is relatively special, so radiotherapy becomes the main treatment of the disease. Some studies have shown that the PinX1 gene may have an inhibitory effect on the tumor, but its mechanism is not clear. Therefore, it is of great significance to study the mechanism of the PinX1 gene inhibiting NPC stem cells.

At present, there are many researches on NPC. For example, Wei studied the genetic and epigenetic changes of a newly isolated nasopharynx cancer 3p21.3 gene RASSF1A (1,2). He examined 4 xenografts, 4 cell lines and 21 primary tumors. He detected methylation of 5'CpG Island promoter in RASSF1A in 4 (100%) xenografts, 3 (75%) cell lines and 14 (66.7%) primary tumors, but not in the normal nasopharyngeal epithelium (3,4). The results showed that 2 of 21 primary tumors (9.5%) had mutations(5). In addition, RASSF1A gene expression was not found in cell lines and xenografts with extensive methylation. After 5 '- aza-2' deoxycytidine treatment, RASSF1A gene re-expression and demethylation were detected in NPC cell lines (6,7). These results suggest that promoter methylation may be involved in the transcriptional inactivation of the RASSF1A gene in NPC (8). The results of this study indicate that the high incidence of RASSF1A gene mutation suggests that it is a key target gene involved in the development of NPC on the 3p21.3 chromosome (9,10). This study has a certain reference, but the sample data is too few, and the results have a certain contingency.

Gorlova used quantitative reverse transcription (RT-PCR) to detect the expression of PinX1 in 73 cases of GC and used microsatellite marker d8s277 to further study the loss of heterozygosity (LOH) in 45 cases (11,12). It was found that in 73 cases of GC, the expression of PinX1 decreased in 50 cases (68.5%) (tumor and normal control < 0.5%) (13-15). In addition, LOH of the PinX1 gene was detected in 15 (33.3%) of 45 GC cases, which was significantly related to the decreased expression of the PinX1 gene (P = 0.031) (16). He also used trichostatin A (TSA) or nicotinamide (NAM) to induce the expression of PinX1 in the human gastric cancer cell line MKN-74 (17,18). These results indicate that the low acetylation of histone H4 in LOH at the PinX1 site and 5'UTR of PinX1 is related to the decreased expression of the PinX1 oncogene (19). Al-

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though the feasibility of this research method is low, it has great reference value.

In order to study the inhibition mechanism of the PinX1 gene on NPC cancer stem cells, this paper set up a control group experiment to study. In the experiment process, after the completion of transfection, cell growth, cell invasion and migration, and cell apoptosis experiments were carried out respectively, and telomerase activity was detected. Among them, after transfection of PinX1 overexpression plasmid into CD133 + cells, the cells grew 1.90-2.17 times in 48h and 72h, which was lower than 1.95-2.48-times of transfection of empty plasmid ($P < 0.05$). On the other hand, after transfection of pinx1sirna in CD133 cells, the cells grew 1.90-2.36 times in 48h and 72h, which was higher than 1.89-2.13 times in transfection of empty plasmid ($P < 0.05$). Therefore, the PinX1 gene inhibited the growth of NPC cancer stem cells.

Materials and Methods

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) often has a pharyngeal recess and posterior parietal mucosa, and the cause of this disease is not clear. At present, some studies suggest that the occurrence of NPC may be related to genetic factors and the EB virus, and environmental carcinogens and autoimmune factors may also be the cause of NPC. At present, radiotherapy is the first choice for the treatment of NPC, including radical radiotherapy and palliative radiotherapy. The incidence rate of nasopharyngeal carcinoma is low, and the incidence is low. The early diagnosis rate is low, and most of them are later diagnosed.

Tumor stem cells

Tumor stem cell (CSC) is a kind of cell with the ability of self regenerate and has the potential of multi-directional differentiation and infinite or infinite proliferation, which may maintain the malignant proliferation, invasion, metastasis and recurrence of tumor, and it may also have the characteristics of asymmetric division and Division (20). In the current academic circles, the theory of tumor stem cells holds that there are one or more groups of cell subsets with stem cell characteristics in tumor tissue, namely CSCs (21). CSCs have the potential of self-renewal and differentiation. Influenced by the microenvironment and the state of the organism, they differentiate into cancer cells with different phenotypes in different tumor growth stages, which can maintain the development and growth of tumor tissue, thus realizing the key role of distant metastasis and growth (22).

Tumor cells have heterogeneity, that is, the formation and penetration ability of cloned cancer cells are different, and the growth speed, differentiation degree, hormone response and sensitivity of anticancer drugs in the process of subclonal development are also different (23). The heterogeneity of tumor cells can only be explained by the theory of tumor stem cells. Under different selection pressures, tumor stem cells differentiate and mature in different functional directions, resulting in the formation of heterogeneity of tumor cell groups. Therefore, tumor stem cells are the main cause of cancer. Eliminating tumor stem cells means eliminating tumors (24,25).

PinX1 gene and telomerase

Telomerase is a kind of reverse transcriptase with its own standard. The composition of telomerase is composed of RNA (TER) carried by telomerase itself, telomerase reverse transcriptase (TERT) and telomerase synergistic protein (htpl). Telomerase DNA can be added at the end of the eukaryotic cell chromosome. Telomerase can lengthen the shortened telomere with limited cell reproductive capacity, thus promoting cell proliferation in vitro. The synthesis of telomerase is a dynamic process, which is regulated in space and time. The scope of human telomerase RNA includes 11 nucleotides, which are complementary sequences of basic sequence units of the human telomere. Therefore, in the absence of DNA mode, it can use its own RNA as a template to obtain a single nucleotide rich in G by adding repeat sequence, extending the oligonucleotide telomere segment, eliminating the composition of DNA telomere, so as to make up for the "end replication" problem of telomere ”The loss of telomere fragment can prevent the decrease of the telomere.

PinX1 is a kind of nuclear protein preserved in human and yeast cells. It has been found that it may interact with telomerase in recent years. The human gene PinX1 exists in chromosome 8p23, which is an area of high-frequency non-uniform elimination in many kinds of tumors, such as liver cancer. PinX1 protein is 328a in length, with one htei binding site at N-terminal (AAL. 142) and one htei binding site at C-terminal (aa254.328). The high expression of pinx1c can significantly inhibit telomerase activity, shorten telomere and inhibit the growth of telomerase-positive tumor cells *in vitro* and *in vivo*.

Selection of research objects and markers

We know that the CNE2 cells of NPC originate from the poorly differentiated squamous cell carcinoma of the human nasopharynx, which has many advantages, such as low differentiation and high proliferation, and the proportion of tumor stem cells is high. On this basis, CNE2 cells of NPC are selected as the research materials in this paper. In addition, CD133 is a glycoprotein on the surface of cells, which has the characteristics of high expression on the surface of hematopoietic stem cells. In the tumor cells with high expression of CD133, the ability of cell proliferation, migration and invasion will be significantly enhanced. Not only that, it also shows strong drug resistance, so it is regarded as a marker in many cancer stem cells.

Preparation of main reagents

In cell culture, we chose DMEM medium for medium selection and preserved it at 40 ° C. when using, we need to add a double antibody solution reserve solution with a concentration of 1 μ L / ml. Our double antibiotic solution is to dissolve penicillin and streptomycin in 0.9% sterilized normal saline for configuration, and after configuration, it will be repackaged at 40 ℃. In addition, the CCK8 experimental detection working solution is prepared by mixing 20 μ l CCK8 original solution + 100 μ L cell culture medium, field configuration, field use, and cannot be placed for too long.

Cell culture

In the first step of cell culture, the water bath pot is preheated to 37 ℃, and the ultra-clean worktable surface

irradiated by ultraviolet for 30 minutes is wiped with 75% alcohol, and then the cell culture bottle is taken out, which sterile operation in the operation process. In the second step, we need to open the bottle cap, suck out the old culture solution, and then wash the cells with PBS, once or twice. Third, we need to add trypsin solution and wash the bottom of the cell dish gently. In the fourth step, we use the pipette to suck and put it up and down several times to break up the cell mass. After the mixture is even, we supplement 3N (N is the number of subculture bottles) ml culture medium and then transfer it to a new culture bottle according to the proportion. The last step is to put it into a C02 incubator. The culture condition is 5% concentration of C02. The humidity is saturated and the temperature is 37 ℃.

CCK-8 detection of cell growth

We need to carry out cell growth detection. In this process, the CD133 + cells are respectively transfected with PinX1 overexpression plasmid, and the CD133 - cells are respectively transfected with PinX1 siRNA and its corresponding NC siRNA. After the transfection, the cells were inoculated into 96 well plates respectively, and the growth of cells was detected by the CCK-8 method and recorded.

Cell invasion

Cell invasion adopts the method of cell chamber invasion. The transfected cells are respectively inoculated into the Transwell cell chamber for cell culture. The process lasts for 48h. The culture conditions are strict, the temperature is 37 ℃, and the concentration of C02 is 5%. The next step is to carry out cell staining. After the staining, count the cells, observe and take photos with a microscope, and randomly take four fields for cell counting. In order to eliminate the chance of data, we will conduct three experiments.

Cell migration

In the cell migration experiment, we also chose the method of cell migration. After the cells were transfected, they were inoculated into 6-well plates and cultured for 48 hours. Similarly, the conditions of the incubator were very strict, the temperature requirement was 37 ℃, and the concentration of C02 was 5%. After that, cells were stained to facilitate observation. After the staining, the cells can be counted. In this experiment, we also repeated three times to prevent the data from being inaccurate.

Apoptosis

In the experiment of apoptosis, we choose the logarithmic growth CD133 + cells and CD133 - cells, inoculate them on the 6-well culture plate and then deal with them after transfection. During the process of treatment, we strictly follow the relevant standards. After the treatment, we stained the cells and put the samples in the flow cytometer 15 minutes later to detect the apoptosis rate.

Telomerase activity assay

In this experiment, we take the logarithmic growth stage cells, inoculate them into six hole culture plate with L × L06 hole, and transfect them respectively 24 hours later. After 48 hours of transfection, we used stretch PCR to detect telomerase activity.

Data processing

The data in this paper are processed by spss21.0 software and tested. Among the data, $P < 0.05$ is statistically significant, that is to say, significant.

Results

Analysis of cell growth experiment results

In CD133 + cells, we transfected PinX1 overexpression plasmid, and in CD133 - cells, we transfected pinx1sirna and NC siRNA and then tested the cell growth through CCK-8 experiment, the experimental results are shown in Figure 1.

It can be seen from Figure 1 that after transfection of PinX1 overexpression plasmid in CD133 + cells, the cells grew 1.90-2.17 times at 48h and 72h, lower than 1.95-2.48 times of transfection of empty plasmid, at this time, $P < 0.05$. On the other hand, after transfection of pinx1sirna into CD133 cells, the cells grew 1.90-2.36 times in 48h and 72h, higher than 1.89-2.13 times in transfection of empty plasmid, at this time, $P < 0.05$. The experimental results showed that PinX1 could inhibit the growth of NPC cancer stem cells.

Analysis of cell invasion results

The results of the cell invasion experiment are analyzed, as shown in Figure 2.

It can be seen from Figure 2 that in the cell invasion experiment, the number of cells in the lower chamber of the CD133- + NC group is 112, that in the CD133- + pinx1sirna group is 172, and that in the CD133- + NC group is 0.65 times of that in the CD133- + pinx1sirna group. In addition, the number of cells in the lower chamber of the CD133+ + vector group was 212, that in CD133+ + over

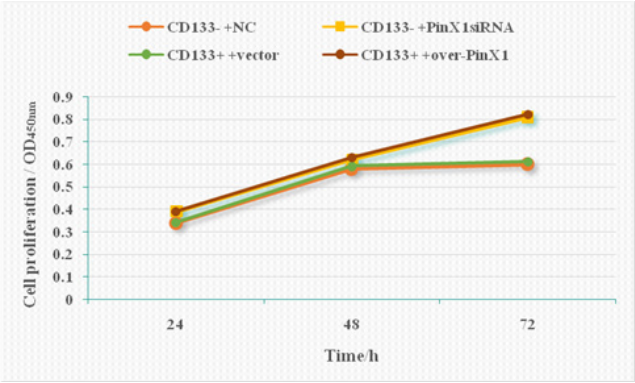

Figure 1. Analysis of cell growth experiment results.

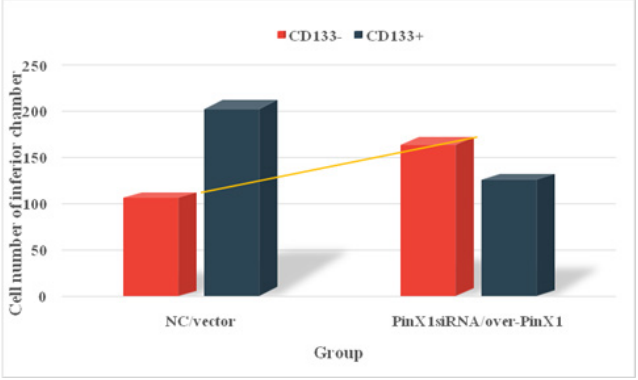

Figure 2. Cell invasion test results.

PinX1 group was 132, and that in CD133+ + over PinX1 group was 0.62 times as much as that in CD133+ + vector group, $P < 0.05$, which further showed that PinX1 could inhibit the invasion of NPC stem cells.

Analysis of cell migration results

The results of the cell migration experiment are analyzed as shown in Figure 3.

It can be seen from Figure 3 that in the cell migration experiment, the number of cells in the lower chamber of the CD133- + NC group is 108, that in the CD133- + pinx1sirna group is 76, and that in the CD133- + NC group is 1.42 times of that in the CD133- + pinx1sirna group. The number of cells in the CD133+ + vector group was 68, that in CD133+ + over PinX1 group was 96, and that in CD133+ + over PinX1 group was 1.41 times that in CD133+ + vector group, Among them, $P < 0.05$. It can be further proved that PinX1 can inhibit the invasion of NPC stem cells.

Analysis of the experimental results of apoptosis

The experimental results of apoptosis were analyzed as shown in Figure 4.

It can be seen from Figure 4 that in the apoptosis experiment, the apoptosis ratio of the lower chamber in the CD133- + NC group is 11, that in the CD133- + pinx1sirna group is 22, and that in the CD133- + NC group is 0.5 times that in the CD133- + pinx1sirna group. The apoptosis ratio of CD133+ +over PinX1 group was 14%, and CD133+ +over PinX1 group was 0.58 times of CD133+ +vector group,. Among them, $P < 0.05$. It can be further proved that PinX1 can inhibit the invasion of NPC stem cells.

Telomerase activity assay

The telomerase activity test results are analyzed, and the results are shown in Table 1.

From Table 1, we can see that the telomerase activity of the CD133- + NC group is 1.001 ± 0.086, CD133- + pinx1sirna group is 0.974 ± 0.046, CD133+ +vector group is 0.928 ± 0.102, CD133+ +over PinX1 group is 0.703 ± 0.086, $P < 0.05$, The results showed that there was a significant difference in the data. It can be seen that the PinX1 gene inhibited the activity of telomerase to produce inhibition on NPC stem cells.

Discussion

The PinX1 gene is an important telomerase inhibitor discovered in recent years. It can inhibit the telomerase activity of some cancer cells, thus inducing cancer cells to reduce and apoptosis. In this paper, it is confirmed that the PinX1 gene can inhibit the tumor stem cells of NPC by inhibiting telomerase activity.

In order to make the experimental results more convincing, we set up a control group experiment, which was transfected in CD133 + cells, respectively transfected PinX1 overexpression plasmid and its corresponding empty plasmid, In addition, PinX1siRNA and its corresponding NC siRNA were transfected in CD133 cells, and then cell growth, cell invasion and migration, and cell apoptosis experiments were carried out.

In the course of the experiment, the experimental results show that PinX1 can inhibit the invasion ability of NPC stem cells. In addition, through the telomerase activity test, it is confirmed that the PinX1 gene can inhibit the tumor stem cells of NPC by inhibiting telomerase activity.

Table 1. Telomerase activity test results.

Group	Telomerase activity	P
CD133- +NC	1.001±0.086	0.011
CD133- +PinX1siRNA	0.974±0.046	
CD133+ +vector	0.928±0.102	
CD133+ +over-PinX1	0.703±0.086	

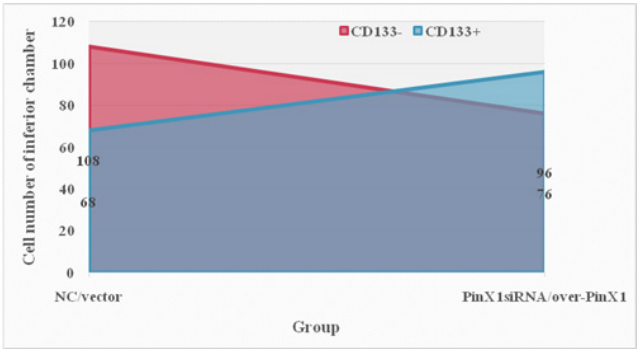

Figure 3. Analysis of cell migration experiment results.

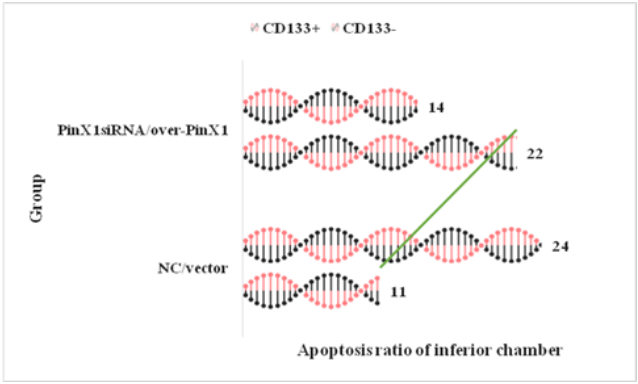

Figure 4. Analysis of the results of the apoptosis experiment.

Acknowledgements

Key Projects of Social Science and Technology Development in Dongguan (No. 201750715001465).

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