The Role of Enhanced Radiosensitivity and Tumor-specific Suicide Gene Vector in Genetherapy of Nasopharyngeal Carcinoma

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Egr-1 promoter/CD suicide gene/hTERT promoter/Survivin antisense oligonucleotides/Nasopharyngeal carcinoma/Gene therapy.

Nasopharyngeal carcinoma (NPC) is one of the common malignant tumors in China. Radiotherapy and chemotherapy are the main therapy methods for NPC. To enhance the specific antitumor effect, a novel vector with radiosensitivity and tumor specificity was constructed in this study, which enables the reduction of dosage of radiation and chemotherapeutic drugs due to its double killing effect. Four DNA elements, Egr-1 promoter, Cytosine deaminase (CD) gene, hTERT promoter, Survivin antisense oligonucleotides were amplified and constructed in pcDNA3.1 vector. CD and Survivin gene expression in CNE-2 cells were detected by RT-PCR. High performance liquid chromatography (HPLC) was employed to determine the transformation from the prodrugs 5-FC to 5-FU. Hoechst33258 staining of the nuclei and methylthiazolyl tetrazolium (MTT) assay were applied to detect apoptosis and cell survivability, respectively. In addition, the anti-tumor effects were examined in vivo by injecting cells with different vectors into nude mice. Our results revealed a notable killing effect of combined treatment with 5-FC and radiation on CNE-2 cells transfected with vectors in vitro. This effect was especially notable on pEC-TS transfected cells, which showed 57\% of cells were killed. In vivo, an obvious suppression of tumor was displayed in pEC-TS group, which was significantly different from other groups (p < 0.05). Consequently, this expression cassette may have a great therapeutic potential for the treatment of NPC.

Nasopharyngeal carcinoma (NPC) is one of the common malignant tumors in pharynx nasalis.\textsuperscript{1} The age of onset of NPC tends to be younger than other cancers, most patients are about 30–50 years old. Radiation therapy is the mainstay of treatment, with chemotherapy used in advanced cases. Though 30\%-50\% of NPC patients can obtain relief by combination radiation therapy and chemotherapy, the 5-year survival rate is only approximately 25\%. Unlike other traditional treatments, gene therapy is a novel therapeutic method for NPC. By the use of combination gene therapy and conventional treatment methods, therapeutic effect of NPC can be further promoted.\textsuperscript{2}

At present, suicide gene therapy is a main strategy for NPC treatment.\textsuperscript{3} Essentially, suicide gene therapy is a form of intratumor chemotherapy that is currently being evaluated in clinical trials. The main mechanism involved is the intratumor delivery of genes encoding enzymes that convert non-toxic prodrugs into toxic metabolites, which will kill the suicide gene expressing cells and the surrounding tumor cells.\textsuperscript{4,5} Among the most intensively studied suicide genes, the most widely used, is bacterial cytosine deaminase (bCD), which can convert thentoxic 5-fluorocytosine (5-FC) to the cytotoxic agents 5-fluorouracil (5-FU). The metabolites of 5-FU, 5-fluoro-20-deoxyuridine 50-monophosphate (5-FdUMP) are incorporated into DNA and RNA, resulting in DNA and RNA chain termination and cell death.\textsuperscript{6,7} Since NPC is highly radiosensitive, Early growth response factor 1 (Egr-1) promoter was introduced in this study to enhance synergistic effects between external irradiation and gene therapy.\textsuperscript{8} It was reported that a satisfactory effect was attained in irradiation-gene therapy researches by the ligation of Egr-1 regulatory sequence and TNF-alpha cDNA.\textsuperscript{9,10} Therefore, double treatments of gene-irradiation therapy for NPC may be generated by combining radiosensitive promoter Egr-1 and suicide gene.

Since the report that antisense RNA can inhibit the gene expression was delivered in 1984, a close attention has been paid to its application in gene therapy. Xiang et al demonstrated an intensive correlation between the expression of
Survivin and the patient’s prognosis.\textsuperscript{11)} Ambrosini \textit{et al} found that Survivin antisense oligonucleotides could inhibit expression of Survivin both in normal and tumor cells, resulting in cell death.\textsuperscript{12)} Thus Survivin may be a great targeting gene or protein for targeted cancer gene therapy. Now the commonly used vectors for gene transfer are lack of selectivity, both tumor and normal cells can be infected with a high risk of damage to normal tissues. Using human telomerase reverse transcriptase (hTERT) promoter to regulate the downstream gene expression is a good strategy for targeted gene therapy.\textsuperscript{13–15)} Koga \textit{et al} and Gu \textit{et al} utilized the hTERT promoter to drive the FADD, bax to induce apoptosis in tumor cells positive for hTERT, but no cytotoxicity was detected in normal cells.\textsuperscript{10)} To reduce the Survivin gene expression in tumor cells and enhance the sensitivity of tumor cells to chemotherapy drugs, we utilize the hTERT promoter to control the downstream Survivin antisense oligonucleotides expression, expecting specific antitumor effect on cell.

To sum up, a novel vector with radiosensitive and tumor-specific promoters was constructed in this study, including CD gene and Survivin antisense oligonucleotides promoted by Egr-1 and hTERT promoter respectively. Under the combination of Egr-1 and irradiation, CD gene may express efficiently, which will convert 5-FC to 5-FU, while Survivin gene expression will be specifically suppressed by hTERT promoter in tumor cells, leading to an enhanced sensitization of tumor cells to 5-FU. With the use of such synergistic effects, the dosage of radiation and chemotherapeutic drugs may be decreased, and antitumor effect may be enhanced. We expect that all of these experiments can provide scientific basis and technology support for clinical trial in gene therapy for NPC.

1. MATERIALS AND METHODS

1.1 Materials

Nasopharyngeal carcinoma cells (CNE-2) were obtained from China Center for Type Culture Collection (Wuhan, China). pEGFP-N1 vector was purchased from Invitrogen (Guangzhou Office, China). Restriction enzyme, polymerase were purchased from TaKaRa (Dalian, China) and Biolabs (Beijing, China). Roswell Park Memorial Institute (RPMI)1640 and Dulbecco modified eagles medium (DMEM) were obtained from Gibco (Shanghai, China). Lipofectamine2000 was obtained from Invitrogen. In vivo-jetPEITM-Gal was purchased from Polyplus transfection (Shenzhen, China).

1.2 Methods

1.2.1 Eukaryotic expression vector Construction

1.2.1.1 Clone of tumor-specific hTERT promoter and initial identification of its function

The hTERT promoter of 300bp core sequence (TERTp) was amplified from human genome with upstream and downstream primers (HindIII and SpfI site were added respectively), and then the products was ligated to EGFP-N1, creating hTERT-GFP vector, which were linearized by NheI digestion and transferred to the CNE-2 cells positive for hTERT and normal human dermal fibroblasts (HDF). The GFP expression was observed under a fluorescent microscope.

1.2.1.2 Radiosensitive and Tumor-specific Suicide Gene Vector construction

mRNA was extracted from Nasopharyngeal carcinoma cells, and reversibly transcribed to cDNA, which then served as template for Survivin antisense strand (200–248 fragment) amplification with primers (BamH I and Age I were added respectively). And the products were ligated to the hTERT-GFP vector. The vector was then cut by Hind III and Not I to generate hTERT-Survivin antisense strand fragments, which was ligated to pcDNA3.1 vector, creating pcDNA3.1-hTERT-Survivin vector (pTS). The yCD gene was amplified from the yeast genome with primers CD-1a/CD-1b, and the Egr-1 promoter was amplified from the mouse genome using primers Egr-1a/Egr-1b. And Egr-CD was amplified by overlap PCR. Then, this fragment was digested with NheI and Afl II, and the product was ligated to pcDNA3.1 vector and pcDNA3.1-hTERT-Survivin vector respectively, creating pcDNA3.1-Egr-CD (pEC) and pcDNA3.1-Egr-CD -hTERT- Survivin (pEC-TS) (Fig.1).

1.2.2 \textit{In vitro} experiments

1.2.2.1 Cell culture and transfection

CNE-2 cells were seeded into 24-well plate at $1 \times 10^5$ cells/well, and maintained conventionally for 24h. Then, $1 \mu$g gene vectors pTS, pEC, pEC-TS were transferred by

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{three_vectors.png}
\caption{The structure diagram of the three vectors}
\end{figure}

\textbf{Fig. 1.} The structure diagram of the three vectors
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2. RESULTS

2.1 Eukaryotic expression vector Construction

2.1.1 Clone of hTERT promoter and activity determination

The 300bp core sequence of hTERT promoter was amplified and ligated to EGFP-N1vector. Then, the vector was verified by enzyme digestion and sequencing (Fig.2). To
show the variation in transcriptional activity of hTERT promoter in tumor and normal cells, gene vectors were transferred into the CNE-2 and HDF cells. 24h later, green fluorescent protein (GFP) expression was observed in CNE-2 cells, but not in HDF cells (Fig.3).

Fig. 2 1, hTERT-GFP vector digested by double restriction enzymes; 2, hTERT-GFP vector digested by single restriction enzyme; M, DNA Marker.

Fig. 3 24h later, after vectors were transferred, about 30% CNE-2 cells expressed the green fluorescent protein by visual examination, and expressed HDF cells were hardly observed.

2.1.2 Radiosensitive and Tumor-specific Suicide Gene Vector construction identification

Gene vectors pEC-TS, pEC, pTS were identified by double restriction enzymes. And the size of fragments were consistent with the anticipated size (Fig.4). All expression cassettes were also confirmed by sequencing.

2.2. Cytotoxicity in vitro experiments

2.2.1 Transfection and positive clones selection

Gene vectors pTS, pEC, pEC-TS were transferred to CNE-2 cells with liposome. Then, the medium was removed and replaced by fresh medium containing 400 μg/ml G418 to select the positive colonies. Cells were incubated for 14 days, and isolated colonies were expanded in a medium containing 200 μg/ml G418.

2.2.2 Survivin and CD mRNA detection by RT-PCR

mRNA extracted from CNE-2 cells positive for pTS, pEC, pEC-TS respectively and wild-type cells, were used as tem-
plate for reverse transcription and PCR to amplify the Survivin and CD gene. The results showed that specific fragments of 398bp (Survivin), 487bp (CD) and 200bp (β-actin) were amplified in the positive cells (Fig.4, Fig.5). And the quantity of Survivin in CNE-2 cells transferred with pTS, pEC-TS were obviously lower than that transferred with pEC.

Fig. 5 RT-PCR results of Survivin: 1, negative control (without template in reaction system); 2, CNE-2 cells transferred with pTS; 3, CNE-2 cells transferred with pEC-TS; 4, CNE-2 cells transferred with pEC; 5, wild type CNE-2 cells; M, DNA Marker. Survivin gene expresses in wild type CNE-2 cells, thus it was detected in all the four groups.

Fig. 6 RT-PCR results of CD gene: 1, DL2000 DNA small Marker; 2, CNE-2 cells transferred with pEC; 3, CNE-2 cells transferred with pEC-TS; 4, wild type CNE-2 cells.

2.2.3 Detection of 5-FU by HPLC
Concentration of 5-FU was measured after 24 hours administration of the prodrugs. In unirradiated CNE-2 cells with pEC showed 51 µg/ml of 5-FU, and it increased up to 90 µg/ml after irradiation. Similarly, 48 µg/ml of 5-FU in unirradiated CNE-s cells with pEC-TS went up to 94 µg/ml after irradiation. While a maximum concentration of 90 µg/ml and 94 µg/ml was detected in that combined 60CO-γ-radiation, which was much higher than the former group. Meanwhile no obvious peak of 5-FU was detected in CNE-2 cells transferred with pTS as well as wild-type cells. (Fig.7)

Fig. 7 shows a maximum concentration of 5-FU determined by HPLC in each group. In CNE-2 and pTS group, no obvious peak of 5-FU was detected both with radiation or not. In pEC and pEC-TS group, 51 µg/ml and 48 µg/ml 5-FU can be detected even without radiation. While much higher concentration (90 µg/ml and 94 µg/ml respectively) appeared after radiation.

2.2.4 Apoptosis detection using Hoechst33258 nucleus staining
CNE-2 cells containing pEC-TS were incubated with Hoechst33258 for 24 hours, and more than 40% of cells induced apoptotic cell death, observed under the laser microscope. Moreover, the number of dead cells in this group was significantly higher than in other groups. (Fig.8)

Fig. 8 Apoptosis detected by Hoechst33258. A, wild-type CNE-2 cells without radiation, B, CNE-2 cells transferred with pEC-TS + radiation + 5-FC, C, CNE-2 cells transferred with pEC-TS + radiation. About 40% apoptotic cells characterized by intensively stained condensed chromatin were displayed in pEC-TS + radiation + 5-FC group, which was much larger than other groups.

2.2.5 Cytotoxicity determination by MTT assay
The cell viability transected with each plasmid was analyzed by MTT. We set the wild-type CNE-2 cells without radiation as the control group, in which the survival cell rate was presumed to be 100%. The survivability of the pTS, pEC, pEC-TS, wild-type, pEC' and pEC-TS' cells under radiation were 90%, 75%, 43%, 84% and 80% respectively; While the corresponding group under no radiation were 97%, 89%, 66%, 100%, 91% and 80% (Fig.9). Thus these results revealed an obvious antitumor effect.

Fig. 9 The wild type CNE-2 cells and therapeutic gene expressing CNE-2 cells were applied to MTT assay. The MTT results showed that the mean cell survivability has striking difference between the radiation and no radiation group in each cell clone (p < 0.05), especially in the pEC and pEC-TS group (75% and 89%, 43% and 66%, p < 0.01). Besides, between the pEC-TS and other groups, the pEC and the pEC' group under radiation, the pEC-TS and the pEC-
TS’ group under radiation or not, there were a very significant difference (p < 0.01), which implied synergic antitumoral effects in this strategy. But the cell survivability between the CNE-2 and pTS group under radiation, the pEC’ and the pEC-TS’ group have no statistical difference. The error bars represent the standard error of the means.

2.3 Animal model construction and evaluation of the antitumor effect in vivo

2.3.1 Observation of tumor growth characteristics

The tumor volumes of each group were measured every 3 days until the experiments were stopped. Growth of tumors formed by transfected cells without radiation was similar to that formed by CNE-2 cells. In contrast, tumors formed by cells with pEC or pEC-TS showed delayed growth, and profound growth suppression was observed when these cells were exposed to γ-rays. (Fig.10).

Fig. 10 Tumor volume was measured every 3 days and its growth curve was draw after experiment stopped. The symbols in right frame of this fig. represent different treatment group. The tumor volumes among the pTS + radiation + 5-FC, the pEC + radiation + 5-FC, the pEC-TS + radiation + 5-FC and the pEC-TS + 5-FC groups were conspicuously different (p < 0.05). Between the PBS and other groups there was a very significant difference (p < 0.01). Whereas between the pEC-TS + 5-FC and the pEC + 5-FC, there was no striking difference displayed. And the tumor volumes among the pTS + radiation + 5-FC, the pEC-TS + radiation, pEC + radiation and the CNE-2 + radiation + 5-FC groups had no statistical difference else (p > 0.05). The error bars represent the standard error of the means.

2.3.2 Detection of 5-FU and gene expression, and pathology result of tumor tissues in vivo

By using HPLC, we detected 5-FU in the serum of the treatment group pEC + radiation + 5-FC and pEC-TS + radiation + 5-FC with the highest concentration of 6 ug/ml and 9 ug/ml respectively. The fragment of therapeutic gene was amplified from tumor tissues in treatment group by RT-PCR. And After the experiments were terminated, the tumor tissues were isolated for HE staining, and the results showed...
that large scale cell death with nuclei disappearing in treatment group, in contrast to no significant cell death were found in control group (Fig.11).

Fig. 11 There are representative sections isolated from tumor tissues of three groups. A, PBS control group; B, pEC + radiation + 5-FC group; C, pEC-TS + radiation + 5-FC group. A large scale cell death with nuclei disappearance in the pEC-TS + radiation + 5-FC group, but no obvious cell deaths were found in control group.

3. DISCUSSION

Radiation therapy is the mainstay of treatment for NPC in clinic, while the suicide gene therapy is a novel but effective strategy in this area. Therefore to explore more potential methods, we established coordinated expression vector consists of radiation responsive promoter, Egr-1, and hTERT promoter. As shown in fig. 6, we demonstrated that Egr-1 promoter efficiently promoted expression of the CD gene. When the CD-expressing cells were treated with 5-FU, the concentration of 51 ug/ml 5-FU was detected. While the cells were exposed to γ-rays, the concentration of 5-FU was increased to 90ug/ml, which resulted in a raise of cell death rate from 11% to 25%. The reason why the content of 5-FU increased in supernatant may be explained for the enhancement of CD transcription activity of Egr-1 promoter induced by 60Co-γ irradiation stimulation. It can be regarded as the direct evidence that the sensitization of CD suicide gene system is under the control of the Egr-1 promoter. Whereas this sensitization was not influenced by the inhibition of Survivin expression. Of course, except for the increase of 5-FU in cells microenvironment, the direct lethal effect of the radiation is another important factor for the raise of cell mortalities.

Telomerase is negative in almost all normal somatic cells. In contrast, it presents high activities in more than 90% of tumor cells, especially in NPC cells. hTERT is a key component of telomerase served as catalytic subunit, which is expressed only in cells and tissues positive for telomerase activity, i.e., tumor and fetal cells. In other words, only hTERT-expressing tumor cells can activate its promoter. Hence, the expression of Survivin antisense oligonucleotides controlled by hTERT promoter can be limited in tumor cells positive for telomerase activity, so that the target therapeutic gene expression can be concentrated into the specific target tumor cells. Our results have checked that Survivin gene expression can be inhibited by its antisense oligonucleotides, but it was unable to kill the tumor cells efficiently both in vitro and in vivo. However, it was reported that 70% of Survivin gene expression was inhibited due to 4003 target antisense oligonucleotides aiming at the 232–251 region of its mRNA, by which a large scale cell apoptosis was induced. According to research studied by Sterngranz R et al., the activity of hTERT promoter may be the key factor to cause this expression difference. Several researches used the hTERT promoter for targeted cancer gene therapy, and their findings indicated the efficacy of target gene was usually hindered by its weak activities.

To educe synergistic effects by integrating various therapy strategies together is a tendency in current gene therapy. In this study, when the cells transfected with pTS, pEC were given 60Co-γ irradiation, the mean cell mortalities was just 10% and 25% respectively, but when the two expression cassettes were combined and treated with the same dosage of 60Co-γ irradiation, 57% of tumor cells were killed; and the trial in animal showed the similar results that the tumors in pEC-TS + radiation treatment group were suppressed effectively. For one thing, after the inhibition of Survivin gene expression, the tumor cells prone to be more sensitive to suicide gene chemotherapy and 60Co-γ irradiation. This may be the reason why there is difference between unirradiated cells only with pEC and those with pEC-TS. For another thing, the bystander effect of suicide gene may be expanded by the induced apoptosis. All of the results revealed that a novel gene therapy vector for enhancing tumor radiosensitivity and specificity was constructed successfully, which have attained a synergistic killing effect on tumor. In brief, in this study an experimental foundation was constructed for the application of this vector in gene therapy for NPC.

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REFERENCES

6. Xia, K., Liang, D., Tang, A., Feng, Y., Zhang, J., Pan, Q.,


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