The Study of the Combined Gene Therapy for Nasopharyngeal Carcinoma in vitro

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ABSTRACT Objective: To investigate the effect of hTERT, C-myc Survivin and VEGF on the human nasopharyngeal carcinoma cell growth, and to investigate the mechanism of short hairpin recombinant plasmid targeting four genes having effect on human nasopharyngeal carcinoma cell growth. Methods: RNAi technology was used to construct a short hairpin recombinant plasmid containing four short hairpin RNAS (shRNA) targeting four genes simultaneously while another plasmid just containing single shRNA against hTERTmRNA, and they were transfected into CNE-2Z cells separately by Liposome; Test groups: Blank control group termed BC Group (no interference) , Negative control group termed NC Group (the plasmid without targeted gene), A group (single-gene plasmid against hTERT), B Group (combined plasmid hitting multiple gene); The transfection was detected under the LSCM; Cell viability was detected by using the MTT; The mRNA and protein expression were determined by RT-PCR and western blot-ting. Results: Compared with that in BC group and NC group, the cell proliferation viability decreased both in A group and B, and which was more lower in B group. The mRNA and protein expression level also reduced significantly, and which was more lower in B group. Conclusions: Four genes are involved in the occurrence and development of nasopharyngeal carcinoma. The combined gene therapy has a better effect on the inhibition of Nasopharyngeal carcinoma cell proliferation than the single-gene interference does.

Key words: Nasopharyngeal carcinoma; RNA interference; hTERT; Combined gene therapy

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Introduction

Nasopharyngeal carcinoma is a common malignant tumor in China with high incidence. Much is known about that its tumorigenesis is a complicated process involved a lot of steps and aberrant genes, but some subtleties of its pathogenesis have gone largely unexplored.The conventional treatment of nasopharyngeal carcinoma is radiotherapy, but always followed by the recurrence of local recidivism, distant metastasis and sorts of complications. In addition, the five-year absolute survival rate is always below 60% [1]. In recent years, RNAi has become a routine tool for studies of cancer gene therapy and has been widely applied in functional genomics research [2]. However, many domestic researches only concerned about single gene interference and had gotten certain achievements without satisfied effect [3]. As the occurrence and development of tumor result from multiple genes mutation, gene therapy technology targeting multiply genes is growing in popularity in the various field of medicine. So far, there is still no combined gene therapy report in the field of nasopharyngeal carcinoma (NPC). This study makes use of gene combinant technology to construct a multiple shRNA expression plasmid with VEGF, c-myc,survivin and hTERT as targeted genes, and then transfected it into CNE-2Z cells. The cells viability of CNZ-2Z were measured by MTT after cells having been tranfected. The PT-PCR and Western-bloting were performed respectively to detect the influence of multiple shRNA expression plasmid on the mRNA level and protein level of the targeted gene. The study aims to discuss the mechansim of this gene therapy in inhibiting NPC cell proliferation and to investigate the effect of VEGF, hTERT, C-myc, Survivin gene in the development of nasopharyngeal carcinoma.

1 Material and method

1.1 Materials

Nasopharyngeal carcinoma cell strain were purchased from Nanjing kaiji biotechnology CO LTD, fetal calf serum were purchased from Gibical, USA, RPMI 1640, 0.25% were bought from Invitrogen, Carrier pGenesil-1 and competence colibacillus DH 5a were purchased from Wuhan Jingsai Biotech group, restriction endonuclease *BamH* Ⅰ, *Hind*Ⅲ, *Sal* Ⅰ, Ligation Mix, SacI, MluI, EcoRI, Eco31I were bought from Dalian baosheng biotech Co LTD. T4 DNA ligase was bought from New England Biolabs group; Gel DNA recove ry kits wer e purchased from Ningbo Zhongding Biotechnology Co LTD; Small amount Extraction Kits and DNA Extraction Kits were bought from Qiagen; Agarose gel recovery kits was bought from Q.BIO gene Co LTD; Lipo2000 was bought from Guangzhou ruibo group; Trizol RNA kit were purchased from Gibeo; ReverTra Ace-α-TM-RT-PCR Kit were purchased from TOYOBO biotechnology CO LTD; The primer was bought from Qiagen.

1.2 Cell cultures

The CNE-2Z cells was cultured in RPMI 1640 culture fluid including 10% fet- al calf serum,100 kU /L Penicillin and 100 mg /L streptomycin, placed in 5% CO_2, 37℃ incubator.

1.3 Plasmid construction

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The targeted gene VEGF, c-myc,survivin and hTERT were found out from the GeneBank (GeneBank Numbers ,in turn, are NM_003376, NM_34815, NM_001168, NM_198253). According to the principles of designing shRNA, a oligonucleotide strand targeting these genes were designed and synthesized. Blast analysis and the SNP analysis were preformed to identify the quality of this plasmid. The target sequence of human VEGF, c-myc,survivin and hTERT were as follows: VEGF:5'-AAACCTCACCAAGGCCAGCAC-3'(1366 ~ 1387bp) . C-myc: 5'-GGCGAA CACACAACGTCTT-3'(1623~1642bp). Surviving: 5'-GCAGTTTGAAGAATTAACCC-3'(394 ~ 414bp). hTERT:5'- GAGCCACGTCTCTACCTTG -3' (3043~ 3062 bp); A target sequence (5'- GACTTCATA AGGCGCATGC -3') was used as a control. The Hairpin chain Oligo DNA, was designed and synthesized. And then the Double-stranded DNA was synthesized after annealing, and was cloned into the pGenesil-1 vector. The constructed plasmid was transformed into to *E. coli* DH5a. to have a multiple shRNA expression plasmid vector.

1.4 Test groups

Blank control group termed BC Group (no interference), Negative control group termed NC Group (the plasmid without targeted gene), A group (single-gene plasmid against hTERT), B Group(combined plasmid hitting multiple gene).

1.5 Transfecting of the plasmid and detection

The CNE-2Z cells with concentration 0.5 x 10^5 / ml was cultured for 48h. The transfection complexs was prepared using plasmid and transfection reagent according to the manufacturer's instruction. (A liquid, made by mixing 100 ul RPMI1640 EP with 2 μl plasmid (2 g/L) in a EP tube, B liquid, got by blending100 μl RPMI1640 EP with 4 μl Lipo 2000, were prepared for making the mixture used for each experimental group). BC group was treated with culture solution as a blank control and NC group was added the negative plasmid as a negative plasmid. The transfection efficiency was measured under LSCM after transfection. A great amount of green fluorescence could be observed in group A and B.

1.6 Detecting cell activity by MTT

The cells were incubated in 96-well plates and performed according to cell transfection procedure mentioned in 1.5. Seeding was perforemed 24h perio to replacing the medium by fresh medium. The 20 μl MTT dye was added to the cells at 20h, 24h, 68h and 92h post-transfection, respectively. , and these cells were incubated to the 37 ℃ 5% CO_2 incubator for 4h. And then each well was added 150μl DMSO that was used to determine the absorbance by Thermo Labsystems.

1.7 Real Time-PCR

Total RNAs were extracted by using TRIZOL and reverse-transcribed into cDNA according to the manufacturer's instruction (TOYOBO biotechnology CO LTD). cDNA of each sample was amplified by real-time reverse-transcription polymerase chain reaction (RT-PCR) using specific primer which were designed and synthesized by TaKaRa Biotechnology (Shanghai, China). The primer sequence were as follow: GAPDH:(forward primer 5'- ACCACAGTCCATGCCATC AC-3', reverse primer 5'-TGACCTTGCCCAC AGCCT-3') ;VEGF: (forward primer 5'- ATGCGG GGGCTGCTGCAATGAC-3', reverse primer 5' CTGGCCTTGG TGAGGTTTGA-3'); C-myc: (forward primer 5'- TGTGGAAAAGAGGCAGGCTC -3', reverse primer 5'- CGCTGCGTAGTT GTGCTGATG-3'); Survivin: (forward primer 5'- GCGCTTTCC T TTCTGTCAAGA-3', reverse primer 5'- CACTTTCTCCGCAGTTTCCTC -3'); hTERT:(forward primer 5'- TGTTTGCGG GGATTCG - GCG-3', reverse primer 5'-CCACGCA GCCATACTCAGG-3). Real-time RT-PCR profile consisted of 10 minutes of initial activation at 95℃ followed by 40 cycles of 5-second denaturation at 95℃ and 30-second annealing at 60℃ and 1 minute extension at 72℃.

1.8 Western blotting

To assess the down-regulation of target gene in CEN-2 cell, the cells was transfected with multiple shRNA expression plasmid and single plasmid, respectively. The protein was collected at 96h post-transfection and stored bellowed 80℃. The equal amount of protein(20μg) were subjected to electrophoresis on sodium dodecyl sulfate(SDS)-polyacrylamide gels and then transfered onto nitrocellulose membranes, which was then blocked with 50 g/L dryskimmilk in TBST(0.05% Tween20) followed by sequential incubation with specific antibodies and their respective secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins were deteced with X-ray film and then the image was scanned by Foretix 1 D software. The down-regulation of protein was calculated accoding to the formula((1-actual grey value/standard value)× 100%.

1.9 Statistical analysis

SPSS12.0 was used to Statistical Analysis. Date was expressed as the mean ± standard deviation (SD). The difference among groups was determined by ANOVA $P<0.05$ was considered statistically significant.

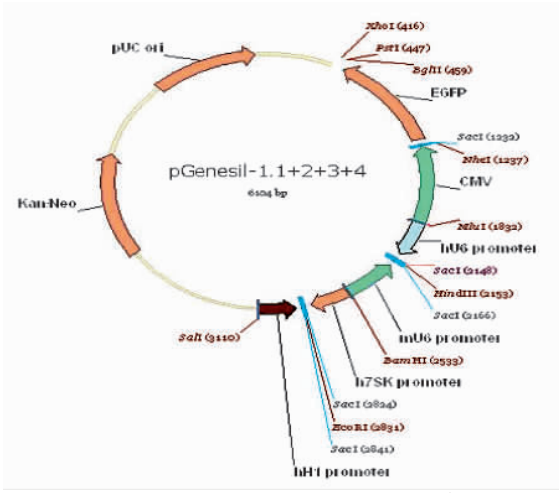
2 Results

2.1 The construction of recombinant plasmid

Plasmid construction was shown in Fig.1.A multiple shRNA expression plasmid vector, targeting four various target genes VEGF,c-myc、survivin and hTERT, was constructed successfully, and was identified by endonuclease cutting and gene sequencing.

2.2 Comparison of the viability of cell proliferation As shown in Table 1

To evaluate the anti-proliferative effect of multiple shRNA expression plasmid, the cell viability was measured using MTT assay and the absorbance was determined, shown in the Table 1. A values at each time points were significantly lower in A group and B group than in BC group with $P<0.05$. Values at each time poin-



mU6 terminator—Sense+Loop+Antisense DNA template2 — mU6 promoter — —
—— h7SK terminator—Sense+Loop+Antisense DNA template3 — h7SK promoter ——
—— hH1 terminator—Sense+Loop+Antisense DNA template4— hH1 promoter — 3'
Transcription direction 3'→ 5'

ts were significantly lower in B group than in A group. The result indicated that the multiple shRNA expression vectors significantly prohibited the cell proliferation compared to the treatment where single shRNA-expressing vector was made.

2.3 48 hours after transfection, the expression of VEGF、c-myc、survivin and hTERTmRNA

Real-time RT-PCR analysis revealed the differences in the expression levels of VEGF、c-myc、survivin and hTERT gene in different groups after 48h of transfection. Quantitative expression of mRNA of different genes in each group 48 h after transfection is shown in Table 2. All the genes mRNA expression were down-regulated in B group compared to BC and A groups. And the mRNA eapression of hTERT in A group was siginicantly lower than control group BC.

2.4 The proteins expression of VEGF、c-myc、survivin and hTERT, refer to Figure 2

To investigate whether the various Plasmid transfecting the cells result in knock down of target genes, VEGF、c-myc、survivin and hTERT , the protein level were assessed at 48h following the transfection. The protein level of hTERT in CNE-2Z cells dropped at (76.3± 4.1)% in A group, while the protein levels of VEGF、c-myc、survivin and hTERT declined simultaneously, at (66.95 % ± 2.42)% 、(62.89 %± 2.97)%、(79.48 % ± 1.92)% and (79.60 % ± 1. 87)% separately in B group. The expression of all protein significantly decreased in B group , and the expression of hTERT protein declined in A group(Fig.2).

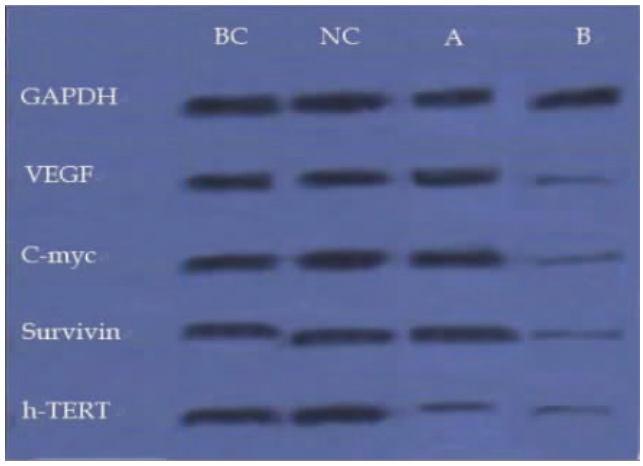


Fig 2 The expression of VEGF、c-myc、survivin and hTERT proteins

Table 1 Comparison of the activity of cell proliferation

Group	n	24h	48h	72h	96h
BC	5	0.499± 0.012	0.786± 0.012	1.208± 0.010	1.352± 0.016
NC	5	0.468± 0.010	0.744± 0.016	1.141± 0.012	1.312± 0.022
A	5	0.352± 0.021△	0.499± 0.020△	0.649± 0.016△	0.738± 0.019△
B	5	0.275± 0.015△*	0.352± 0.019△*	0.371± 0.015△*	0.379± 0.023△*
F		271.438	460.740	374.692	342.134
P		P<0.01	P<0.01	P<0.01	P<0.01

Table 2 The expression of VEGF, c-myc, survivin and hTERTmRNA48h after transfection

Group	N	VEGF	c-myc	survivin	hTERT
BC	3	0.873± 0.025	0.702 ± 0.030	0. 703± 0. 024	0. 859± 0. 124
NC	3	0.863± 0.034	0.692 ± 0. 034	0. 689± 0. 075	0. 824± 0. 034
A	3	0.842± 0.073	0.670± 0.021	0.624± 0.125	0.243± 0.013*
B	3	0.203± 0.063*	0.145 ± 0. 029*	0. 134± 0. 020*	0.203± 0. 021*
F		368.947	761.154	198.457	229.134
P		P<0.01	P<0.01	P<0.01	P<0.01

3 Discussions

Nasopharyngeal carcinoma is a common malignant tumor in China with high incidence. Much is known about that its tumorigenesis is a complicated process involved a lot of steps and aberrant genes, but some subtleties of its pathogenesis have gone largely unexplored. So further investigation of tumor-related gene can better help us study the pathogenesis of nasopharyngeal carcinoma and find effective gene therapy. And RNAi is post-transcipitonal suppression method and artificially induced introduction of double-stranded RNA molecule[4] and it has been applied in variou fields of study. But many researches are about single gene interference which caused limited knockdown of target gene. In this study, we attempted to improve the efficiency of RNAi by developing a multiple shRNA expression plasmid vector targeting four genes.

The Telomeres, the DNA-protein complexes at the ends of eukaryotic chromosomes, are necessary for chromosomes stability through protecti ng th e genome f rom deg radation. end-to-end fusion, and inappropriate recombination[5]. It has been identified that the length of the telomere is highly correlated with tumor formation, while the equilibrium of telomere length is maintained by telomerase which is reactivated or up-regulated in about 85% tumor cells [6]. The telomerase is composed primarily of two subunits, TER and catalytic subunit (TERT). And studies show that telomerase activity is correlated with TERT expression[7], and even the level of hTERT messenger RNA(mRNA) is found to be in direct proportion to telomerase activity in some particular cell types[8]. It is reported that most nasopharyngeal cancer cells persistently express high levels of telomerase activity, whereas a healthy nasopharyngeal epithelium rarely displays. C-myc, is an important transcription factor that involving in regulating cellular proliferation, cell growth, and differentiation, Studies show that its up-regulation may be responsible for promotion of the rate of regional lymph node metastasis and five-year absolute survival rate of nasopharyngeal carcinoma[9]. VEGF (Vascular endothelial growth factor), the most powerful angiogenic factor, has an important effects in tumor angiogenesis and was expressed in many malignant tumors. And its expression positively correlates with the development of nasopharyngeal carcinoma. It is reported that VEGF performing the function of promoting Tumor growth and metastasis by acting on Flt-1and KDR receptors expressed on endothelial cell of new vasculature[10]. Survivin is, a tiniest member of inhibitors-of-apoptosis gene family, is ' turn off ' gene expressed in all the most common cancers but not in normal differentiated adult tissues. Study has shown that survivin is highly expressed in nasopharyngeal carcinoma as well as in the lung, breast tumors and other tumors [11].

We know that the development of tumor results from multiple gene mutation. These studies show that the hTERT expression level can be up-regulated by c-myc over-expression, which is correlated with telomerase activity that is main factror in stabilizing telomere structure [12]. And Survivin may promote Cell transformation function through c-myc and cyclin Dl [13], and also can boost gene transcription by up-regulating C-myc expression leading telomerase to reactivation. Therefore, the key to inhibit telomerase activity is to restrain the expression of survivin leading to the reduction of C-myc expression accounting for the recession of hTERT expression. It also can directly reduce the level of c-myc expression.'said Endoh[14]. These support that there is a complex interaction network regulating the develpoment of nasopharyngeal carcinoma. RNA interference (RNAi) technology has become a novel tool for silencing gene expression in tumor cells ,but previous studies mainly involved in single gene interference and had gotten certain achievements without satisfied effect. Whether combined gene therapy is more effective than single gene therapy to cause gene silencing, to inhibit tumor cell proliferation and metastasis, and enhance anti-tumor effect?

In the present study, we attempted to improve the efficiency of gene siliencing of short hairpin RNA (shRNA), we inserted multiple shRNA expression sequences into a single plasmid vector, which targeting four genes VEGF, hTERT, C-myc, Survivin. Another single shRNA-expression vector against hTERTmRNA was constructed as a control. The result shows that we successfully constructed a multiple shRNA expression plasmid vector and transfected it into CNE-2Z cells. The MTT assay way performed to measure the cells viability of CNZ-2Z after cells having been tranfected. The results showed that multi-gene plasmid vector exhibited higher efficiency tumor cell proliferation inhibition compared with single-gene plasmid. In addition, The PT-PCR and Western-bloting were performed respectively to investigate the influence of multiple shRNA expression plasmids on the mRNA level and protein level of the targeted gene. The result also indicated that the expression of VEGF, hTERT, C-myc and Survivin mRNA and protein was decreased simultaneously after multiple shRNA expression vector transfection and the expression of hTERT mRNA was surprisedly declined more significantly than in control group treated with single-gene plasmid vector. Our study demostrates that genes VEGF, Htert, C-myc and Survivin play an important role in the development of nasopharyngeal carcinoma. The combined gene therapy has a better effect on the inhibition of nasopharyngeal carcinoma cell proliferation than the single-gene interference does, which is consistent with available experimental results [15,16]. Multiple shRNA expression plasmids have shown prospective in gene therapy. However, whether there is a synergy effect among different genes in nasopharyngeal carcinoma and how to find an effective combination of genes way is still unknown. Such mechanisums have gone largely unexplored.

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鼻咽癌联合基因治疗的体外研究

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摘要 **目的**:探讨人端粒酶逆转录酶(hTERT),癌基因蛋白(C-myc),存活素(Survivin),血管内皮生长因子(VEGF)基因对人类鼻咽癌细胞生长的影响,以及同时编码四个基因的短发夹重组质粒对人鼻咽癌细胞生长抑制作用及机制。**方法**:利用基因重组技术构建一个同时靶向作用四个基因的短发夹双链 RNA(shRNA)真核表达载体和靶向单独作用 hTERTmRNA 的 shRNA 真核表达载体,脂质体法转染人 CNE-2Z 细胞;试验分组:空白对照组 BC 组(不进行干扰),阴性对照组 NC 组(加入阴性质粒),A 组(hTERT 单基因质粒组),B 组(多基因联合质粒组)。激光共聚焦显微镜观察转染情况;MTT 法检测细胞增殖活性;RT-PCR 和 Western blot 法分别检测转染后细胞内各基因 mRNA 和蛋白表达情况。**结果**:MTT 法检测,与 BC 相,NC 组相比,A 组和 B 组的细胞增殖活性均降低,与 A 组相比,B 组的增殖活性降低更显著;RT-PCR,Western blot 法,A 组和 B 组 mRNA 和蛋白表达水平均降低,B 组降低更显著。**结论**:四个基因共同参与了鼻咽癌细胞的发生和发展。多个基因的联合干扰与单基因干扰相比,能更高效下调各基因蛋白在鼻咽癌细胞的表达水平,更好抑制鼻咽癌细胞的增殖。

关键词:鼻咽癌;RNA 干扰;hTERT;联合基因治疗
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