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The Promising Anti-Tumor Impact of Newcastle Disease Virus Expressing IL-2 and P53 Genes in Many Cancer Cell Lines *In vitro*

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Abstract: Recombinant Newcastle disease virus (rNDV) has shown an anticancer effect in preclinical studies, but has never been tested in a lung cancer models. In this study we explored the anticancer activity of genetically modified NDV expressing IL-2-P53 (rClone30–IL-2-P53) in lung cancer model. We have cloned IL-2 and P53 genes and inserted them in the viral genome of New Castle Disease Virus to create a genetically modified rNDV- IL-2-P53 virus and tested the anti-tumor activity of the new virus in vitro on different types of cancer cell lines by MTT assay. TheIL-2 and P53 gene were successfully cloned and inserted into the viral genome by using a Mlu I and Sfi I endonucleases, viral vector was constructed correctly and successfully; sequencing results also showed that the recombinant plasmid was successfully constructed resulting in the formation of rClone30 NDV expressing both IL2 and P53 gene. In this study, P53 and IL-2 gene were successfully constructed into the NDV genome, by the use of reverse genetics technology, then successfully rescue of all recombinant rNDVclone30s and got high titer recombinant viruses.

Keywords: rNDV, IL-2, P53, lung cancer, MTT assay.

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Introduction:

The currently used conventional therapies which include surgery, radiation therapy and chemical treatment are the first line in treating cancer, these therapies although showed some success in controlling tumor growth, but they have failed to solve the metastasis problem and causing many severe side effects to the patient such as drug sensitivity, nausea, vomiting, hair loss and Leucopenia, radiotherapy, mainly used for the treatment of systemic proliferation of tumor, such as esophageal cancer, lung cancer, throat cancer, nasopharyngeal cancer, and skin cancer. But once the cancer has spread to tissues, radiotherapy is not up to the desired therapeutic effect.

Using the biological ways to treat cancer- gene therapy, immune therapy, viral therapy, which have been developed recently-is relatively a novel approach depending on the activation of the body's immune system to fight cancer cells (cancer immunotherapy), it has become a major cancer treatment technology(1,2).

Virotherapy is a promising developed treatment using biotechnology to convert viruses into therapeutic agents by reprogramming viruses to treat diseases. Virotherapy includes three main branches: anti-cancer oncolytic viruses, viral vectors for gene therapy and viral immunotherapy, virotherapy can also

refer more broadly to the use of viruses to treat certain medical conditions by killing pathogens.

Many studies have reported the oncolytic activity of NDV towards human tumor cells. The interesting aspect of NDV is its ability to selectively replicate in cancer cells. Some of the studies have undergone human clinical trials, and affirmative results were obtained, therefore, NDV strains can be the potential therapeutic agent in cancer therapy. However, investigation on the therapeutic perspectives of NDV, especially human immunological effects, is still ongoing.

IL-2 is a pleiotropic single chain peptide structure cytokine coding the immune system and produced by T lymphocytes and to a less extent by natural killer (NK) and dendritic(DC) cells (3). its function is regulating and activating white blood cells (usually lymphocyte) which are responsible for immunity, it plays a pivotal role in distinguishing between exogenous genes and self-genes, and in magnifying natural killing (NK) cytolytic activity, promoting antibody production, and inducing the proliferation and differentiation of B cells (4,5). When a helper T cell binds to APC through CD28and B7, CD4 + cells produce IL-2. This cytokine itself supports the proliferation and differentiation of any cell that has high-affinity to IL-2receptors (6). IL-2 plays essential roles in key functions of the immune system, tolerance and immunity, primarily via its direct effects on T cells, plus promoting the differentiation of T cells into effector T cells and into memory T cells when the initial T cell is also stimulated by an antigen, thus helping the body fight off infections (6). It also has a key role in enduring cell-mediated immunity through its role in the development of T cell immunologic memory, which depends upon the expansion of the number and function of antigen-selected T cell clones (6,7).

Because of high correlation between P53 gene and cancer, the study of P53 gene in cancer treatment is becoming a hot spot. Based on the P53 gene inactivation mechanism, a number of different strategies for the treatment of tumors have been developed, since inactivation of P53 gene is the most common event in human cancers, thereby activating the P53 gene is believed to effectively block the formation of tumor entities. In fact, a large number of experimental data show also confirmed P53 gene inactivation and promote tumor cell immortalization(8). In a large number of experimental results show, P53 gene activation is an effective anti-cancer strategies, P53 gene activates negative tumor cells. Therefore, there are some strategies designed to restore tumor cell P53 gene and P53 pathway activation. PRIMA is the use of this strategy of innovative medicines; it can change with the wrong idea of P53 mutations in tumor cells, so that the P53 restore normal function(9-11).

In this study, we have introduced sequences encoding P53 and/or IL-2 into the genome of NDV Clone30 strain at the position between F and HN genes, resulted in recombinant rNDVs (rClone30-P53-IL-2). Our results showed that rClone30sencoding IL-2-P53 was successfully constructed.

Materials and methods:

Materials:

Cell lines, plasmids, and viruses:

RClone30 transcription of the full length of cDNA plasmids, rCLone30,

and helper plasmid pBR-NP, pBR-P, pBR-L were constructed and rescued in the laboratory. The plasmids of prClone30, pTM-NP, pTM-P, and pTM-L were cloned from the NDV lentogenic strain rClone30 (pBrClone30) by our laboratory. The nucleotide sequence of P53 gene was identified by sequence analysis and compared with the reported P53 gene [GenBank: 82395019]. The plasmid pMD18-T-IL-2 was kept in the laboratory.

Chick Embryos:

9-11 day specific pathogen free (SPF) chick embryos were used for virus propagation).

Primers:

The IL-2 (462 bp) and P53 (1954 bp) genes were cloned into pMD18-T simple vector by polymerase chain reaction (PCR) method. For amplification of IL-2 and P53, IL-2and P53 genes primers were synthesized by Invitrogen Corporation. As follows:

Table (1): The primers for gene amplification

primer name	Primer sequence (5′−3)
Primer 1(P1)	ATGGAGGAGCCGCAG
Primer 2(P2)	TCAGTCTGAGTCAGGCCCTT
Primer 3(P3)	GGTTAACCGCCACCATGTACAGGATGCAACTCCTGTCT
Primer 4(P4)	CGACGCGTCGCAATTAAGTCAGTGTTGAGATGATGCT
PIL2F '	GGCCTGAGAGGCCTTAAGAAAAAATACGGGTAGAAGGCCACCA TGTACAGGATGCAACT
PIL2A '	GGCCTCTCAGGCCTTAAGTCAGTGTTGAGATGATGCT
Corresponding restriction sites as:	GTTAAC: HpaI; ACGCGT: MluI; GGCCTGAGAGGCC: SfiI GCCACC the Kozak sequence
Viral RNA reverse transcription primer	5'-CACAGATGAGGAACGAAGGT-3'

Methods:

Cloning and sequence analysis of IL-2 gene and P53 gene:

Total RNA extraction from human tissue:

Take about 100mg of placental tissue, slowly add liquid nitrogen into a pre-cooled mortar, and quickly ground into a powder. Tissue powder was put into a pre-cooled Eppendorf (Ep) tube then 1 ml of cold Trizol reagent was added. The reagent tube (EP) was mixed by upside down thoroughly, then 200 µl phenol – chloroform was added to the mixture (chloroform: phenol = 5:1), shacked well and centrifuged at 4℃ to 12000 rpm / min for 10min. After collecting the supernatant, equal volume of isopropanol reagent (already pre-cooling) was added, incubated in ice for 10min, at 4℃ condition and centrifuged at 12000 r / min speed for 10min. Supernatant solution was removed , ml of ice-cold 70% ethanol was added and centrifuged at 12000 r / min for 5min, this step was repeated three times by ethanol. After completion of the washing, samples were dried at room temperature for 5min. 20 µl sterilized DEPC water was added to each EP tube to dissolve RNA, the remaining was saved in 75% ethanol and placed in -80℃

refrigerator for later use and according to TaKaRa company.

Human tissue cDNA synthesis:

RNA described in the above step was used as a template, based on Oligo (dT) 18 as primer according to the reverse transcriptase (M-MLV) manual cDNA synthesized the first chain reaction system is as follows:

Oligo (dT)18 1.0uL, RNA template 5.0u, DEPC-H2O 5.0uL was incubated at 70°C water bath for 5 min, then in ice for 5 min, and then the followings were added, RNasin 1.0uL, 5xM-MLVRT buffer 5.0uL, dNTPs 5.0uL, DTT 2.0uL, M-MLVRT 1.0uL.

Mixture was incubated at 42ºC in water bath for 2h, then at 70ºCin water bath for 15 min.

Amplification of human IL-2 gene by PCR cloning:

According to the IL-2 gene sequences available on GenBank (serial number NM_000586.3), use primer design software to design primer 5.0 Prime1 and Prime2 IL-2 gene cloning.

cDNA templates obtained by reverse transcription, PCR steps are 95ºCfor 5min,94ºCfor 1min,54ºCfor 30s,72ºCfor 1min, cycle for 30 times, system are as follows:

XPCR buffer 2.5uL, dNTPs 2.5uL, Template 2.0uL (10ngT), Prime1 1.0 uL(10p ml), Prime2 1.0uL(10p mol), Taq enzyme 1.0uL, Deionized water 15uL (Steps according to TANGEN plasmid small Kit company).

Purification of PCR products:

20 µl of PCR products was taken (concentration about 100 ng/µl) purified according to Axygen step purification of PCR Purification Kit instructions: the PCR reaction mixture, 3 times the volume of Buffer PCR-A was added, and mixed. 2 mL micro-centrifuge tube was taken, 500uL balanced solution added, then centrifuged at 12,000 r/m for 1 min, and the supernatant then discarded. The step was repeated twice, the mixture in step 1 was joined on the joined columns and balanced, static for 1min, and centrifuged at 12,000 r/min for 1 min. Supernatant was discarded, and 700µL Buffer W1 was added, then centrifuged at 12,000r/m for 1 min. Supernatant was discarded; 700 µl Buffer W2 was added, and centrifuged for 1min, repeated twice.

The adsorption column of PCR products was transferred to a clean 1.5 mL centrifuge tube, tube film central loft was prepared, 25~30 µl of deionized water was added, left for 1 min at room temperature and centrifuged at 12,000 r/m for 1 min to elute DNA**.** 1 µL of purified product was electrophorsed in 1% agarose gel and then stored at-20°C ready for use.

Purification of PCR products and ligation of cloning vectors:

The purified fragment obtained was cloned to the vector pMD18-T according to the instruction manual. Ligation reaction as follows:

5.0 ul of purified PCR product was mixed with 1.0uL PMD-18-T Vector and 4.0ul solution. Total volume is 10ul, mixed well, and incubated at 4℃ overnight for ligation according to TANGEN plasmid small Kit Company.

Transformation of recombinant plasmid cloning:

The ligation product will be fully transformed into E.coli DH5α

competent bacteria, specific transformation steps are as follows:

The DH5 competent bacteria stored at -80℃ refrigerator was removed from the fridge, and left to stand on ice to melt quickly. After the competent bacteria being thawing, the ligation product (10uL) was added, and prepare another tube to use as a negative control. The two competent tubes were placed on ice for 30 min, then placed in 42℃ water bath for 90 sec heat shock, then quickly returned to the ice, stand for 3-5min.

In a laminar flow hood, the mixture was added to 200 μl of sterilized LB broth appeal to each tube and then fixed to the shaker frame at 37℃ in spring gentle shaking for 1h**.** An hour later, in a laminar flow hood, the above transformation was taken and mixed well, applied to 400μl containing 100 μg / ml Amp plates of LB solid medium, apply respectively DH5α containing 100 μg / ml on LB solid medium and non-Amp Amp as a control, alcohol lamp burner glass coating bar coating uniformity was used, placed first at 37℃ incubator for 30-60min, until the liquid has penetrated into the surface of the culture within the group, then put upside down at 37℃ incubator for overnight.

Screening of positive clones:

A few individual colonies and were randomly picked and inoculated in LB medium containing 100 μg g/ml Amp, incubated at 37°C in liquid medium for overnight, bacterial plasmid was collected, plasmid extraction steps according to TANGEN plasmid small Kit company (centrifugal type) instructions are as follows:

Bacteria was collected, 1.5 ml liquid was taken into join Ep tube, centrifuged at 12,000 r/min for 1 min, the supernatant was discarded and repeated 3-5 times, 250 μl solution P1 added to the cell precipitated in the centrifuge tube, use pipette to suspend the precipitated bacteria, then 250 μl solution 2 was added, gently upside down 4~6 times to sufficient cell lysis. Finally 350 μl solutions 3 added in centrifuge tube, then gently upside down 6~8, mixed well and centrifuged for 15 min at 12,000 r/min, the supernatant was carefully transferred with a pipette into the adsorption column, centrifuged at room temperature for 3 min, at 6000 r / m for 3 min, the waste was discarded in a collection tube, re-put the adsorption column into the collection tube. 700 μl rinse PW was added to the adsorption column, centrifuged at 12,000 r / m for 1 min, and discarded the waste collection tube. 500 μl of the rinsing solution was added to adsorption column PW, centrifuged at 12,000 r/m for 1 min, then the waste collection tube was poured off. the column back was putted into the collection tube, centrifuged at 12,000 r/m for 2 min in room temperature, dried at room temperature. the column was placed into a clean EP tube, perpendicularly to the central adsorbed film dropped 30 μlabout 70℃ sterilized deionized water, incubated at room temperature for 5 min, centrifuged at 12,000 r / m for 2 min to collect the plasmid solution into a centrifuge tube. 1% agarose gel was used for electrophoresis results, the plasmid was placed -20℃ ready for use.

Identification of recombinant plasmid:

For convenience of description, the recombinant plasmid was named pMD18-T-IL-2, digested with internal Kpn I restriction enzyme to pMD18-T-IL-2 single restriction enzyme, incubated at 37℃ for 2h in 10μl enzyme digestion system:

Kpn I	1.0uL
Buffer 1 (NEB)	2.0 μL (1.0) μl
PMD18-T-IL-2	1.0 μL
ddH_2O	6.0uL

After digestion, use a 1% agarose gel electrophoresis to get the results.

Cloning of human IL-2 gene sequencing and result analysis:

The identification of the correct recombinant plasmid was sequenced by the Nanjing Company PBL. Use DNAMAN software for sequence analysis of sequencing results.

rNDV-P53-IL2 genome construction of full-length cDNA:

Preparation of NDV genome vector:

The Laboratory saved rNDV-P53 plasmid was double-digested fragment, recovered vector prepared, digestion system is as follows:

rNDV-P53) 20.0μL (20μg), Mlu I 10.0μL, Sfi I 10.0μL. K buffer 20.0μL. ddH2O 140.0uL

The enzyme reaction system was incubated in 37℃ for 2.5 hours. Followed by gel digestion products of plastic recycling purposes then recovered around 18,000 BP fragment. place 200 μl (~ 20 μg) enzyme products on 1% agarose gel electrophoresis, after electrophoresis is over, find the target in a UV light irradiation, using rubber cutting knives, cut approximately 18,000 BP of the target gene, weigh the glue. For recovery steps to Axygen Company Gel Extraction Kit, 300μl was added to each 100mg agarose of DE-A solution, the agarose was completely melted in a water bath on 70℃, during the solubilization was shocked 2-3 to colloidal melt. When agarose is completely melted, DE-B in an amount of 1/2 DE-A liquid volume was added, mixed well; moved liquid into equilibrated adsorption column, centrifuged at 12,000r / min for 3min, The adsorption column was set up into the collection tube, 500 μl W1 liquid was added to the adsorption column, centrifuged at 12,000r / min for 30sec, The liquid was drained out of the collection tube, the adsorption column was placed into the same collection tube. 700μL W2 was added, centrifuged at 12,000r / min 30sec, in the adsorption column, the liquid poured in the collection tube, the adsorption column into the same collection tube, centrifuged at12000r / min for 2min. The column placed into a clean 1.5ml Ep tube vertically in the center of the adsorbed film, 40μl sterile deionized water was added, incubated for 1min, centrifuged at 12000r / min for 1min, in bottom of the tube is the desired solution of DNA fragments. 1μl of sample was taken and use a 1% agarose gel for electrophoresis and the stock stored at -20℃ for long-term use.

Preparation of IL-2 fragment:

Certified correct positive recombinant plasmid pMD18-T-IL2, digested fragment recovered, Mlu and

Sfi I digested System (200µl) is as follows.

PMD18-T-IL-2 20.0µL (20µg), Sfi I 10.0µL, Mlu I 10.0µL, K buffer 20.0µL, ddH2O 140.0uL

At 37℃ the digested reaction system for was incubated 2.5 hours. Then gel digested product was recovered fragment of about 540 bp. Gel Extraction is as step above**.**

Recycling of IL-2 fragment and ligation with pBrClone30 vector:

After the purification of gel extraction product, IL-2 gene transcription was ligated to the pBrClone30 vectors, connection system is as follows:

PBrClone30 carrier 1.0 µl（10 ng）, Purification of plastic recycling products 3.0 µl（30 ng）, T4 ligase (NEB) 0.5 µl, T4 Ligation Buffer 1.0 µl, Sterile deionized distill water 4.5 µl.

Total volume was 10 µl system, mixed well then incubated at 16ºC overnight for ligation, product will be ligated to all of STBL2 cells, then transformation step at temperature 30℃, number of revolutions 70 r/m for 16h, and steps were repeated.

rNDV-P53-IL-2 plasmid:

The IL-2 fragment was purified by gel extraction using T4 DNA ligase rNDV-P53-MluI-SfiI vector, the ligation reaction of total volume (10 µL) is as follows**:**

rNDV-P53-MluI- Sfi support 1.0µL, IL-2 fragment 3.0 µL, T4 Ligase (NEB) 0.5µL, T4 Ligase Buffer 1.0µL, ddH2O 4.5µL.

The total 10µl system prepared, and followed by sufficient mixing at room temperature for overnight for ligation, ligated product was completely transformed by E coli. DH5α competent bacteria and a control group, applied on solid LB medium plate containing 100µg / ml Amp's, incubate at 37℃ culture for 12h concrete steps. Several single colonies were picked and inoculated into LB liquid medium containing 100µg / ml Amp, the amplified cultured was incubated overnight, according to plasmids Axygen company Little mention plasmid kit, the specific reference to step 3.1.7. The extracted plasmid enzyme digestion and PCR identification. Identification system is as follows:

Digestion system:

Plasmid 2.0µL (1µg), Sfi I1.0µL, Mlu I 1.0µL, K buffer 1.0µL, ddH2O 5.0uL.

PCR System:

PCR was prepared and after the identification is confirmed, the product sent to Shanghai Biological Co. to be sequenced. Use DNAMAN software for sequencing results sequence alignment and analysis, and sequenced properly, positive plasmids were stored at -20℃ ready for use.

Results:

Preparation of human tissue cDNA:

After the extraction of total RNA from human placenta tissue samples, total RNA was converted to cDNAby reverse transcription, the quality is good, gel electrophoresis results appears as a diffuse band, (Figure 1).

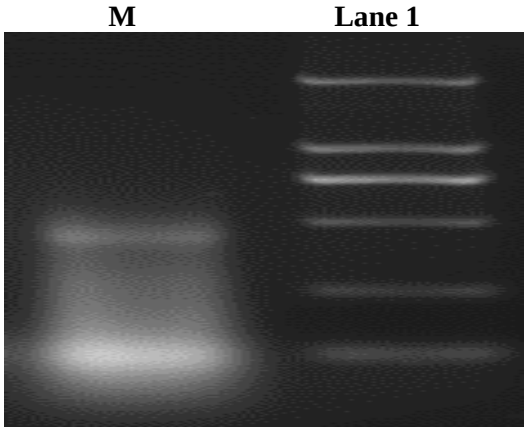

Figure (1): cDNA identification: DL 2000 DNA Marker, Lane1: cDNA (sample volume 5 µl, gel concentration 1.5%, electrophoresis time 90 min.)

Cloning of human IL-2 gene:

Using human IL-2 gene -specific primers, gene upstream and downstream ends of specific primers were designed, MluI and SfiI recognition sequence cut, IL-2 gene was ligated with the NDV genome. Human placenta cDNA used as a template, the human IL-2 gene was amplified, the PCR product used on a 1% agarose gel electrophoresis, the results shown in (Figure 2).

Figure 2 shows , human placental RNA reverse transcription product as a template, cDNA was amplified by a specific band of about 500 bp, the initial determination is what we want human IL-2 gene. Repeat the PCR process and recovered PCR product was connected with pMD18-T vector designated pMD18-T-IL-2, transformed by DH5α E. coli, ampicillin applied to content on LB solid medium 100mg/L. After overnight incubation, single colonies were picked, cultured, plasmids were extracted after identification by PCR, the plasmid positive results sent to sequencing. After sequencing, results were compared with the known sequence of results shown in Appendix I, Human IL-2 sequence alignment, results of sequencing, indicated that the expected gene cloning success.

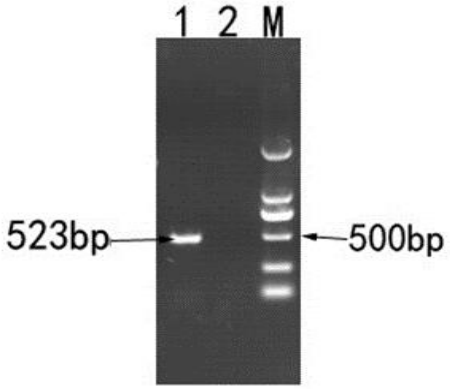

Figure (2): PCR amplification of IL-2 gene, Lane M：DL2000 Lane 1：IL-2genePCRproduct , Lane 2: control(sample volume 5 µl, gel concentration 1.5%, electrophoresis time 90 min.

PCR amplification of IL-2 gene:

PMD18-IL-2 plasmid is kept in our laboratory as a template, and PIL-2F and PIL-2A;PIL-2F' and PIL-2A' two PCR primers sub cloning IL-2 results shown in (Figure 4 and 3). As can be seen from Figure 4 and 3 each of the two primer pairs amplified with IL-2, IL-2 encoding box (489bp) and IL-2' (517bp) gene.

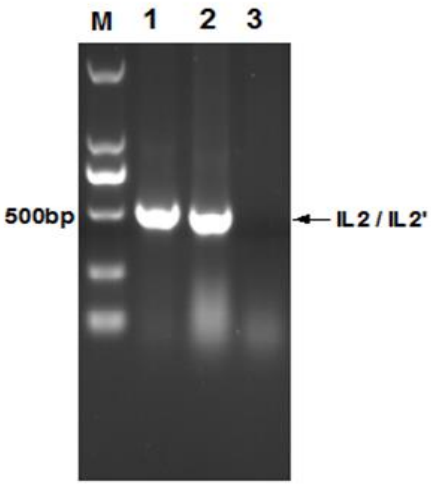

Figure (3): Amplification of IL-2 and IL-2'genes from Plasmid
M：DL2000； 1： IL-2'PCR product; 2： IL-2 PCR product； 3： control (sample volume 5 µl, gel concentration 1.5%, electrophoresis time 90 min).

Purification of PCR product:

PCR products were submitted to 1% agarose gel electrophoresis for gel extraction to obtained PCR product which was in consistent with the target gene size, as shown in (Figure 4).

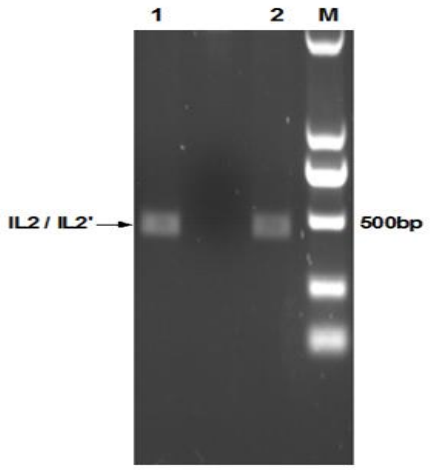

Figure (4): Gel extraction of IL-2 and IL-2' genes
M：DL2000； 1：IL-2 cycling products；2： IL-2 cycling products(sample volume 5 µl, gel concentration 1.5%, electrophoresis time 90 min).

Preparation of PBrClone30 vector:

NDVClone30 strain pBrClone30 genome transcription of the full length cDNA plasmid was double digested with HpaI, MluI, to get 18000bp fragments, after gel extraction purification, store at-20℃ ready for use, as shown in (Figure 5).

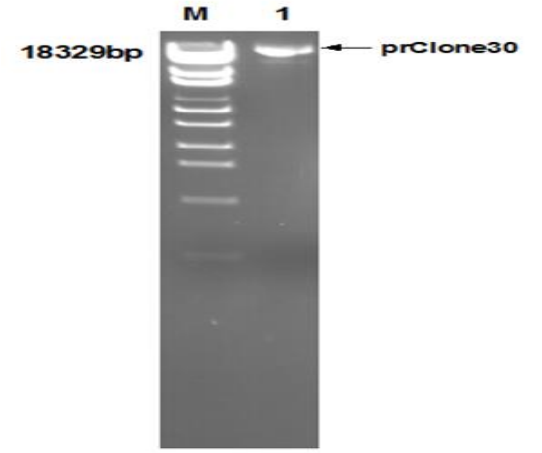

Figure (5): Gel extraction of prClone30
M：λ-EcoT14 1：prClone30 vector cycling product(sample volume 5 μl, gel concentration 1.5%, electrophoresis time 90 min).

Construction of NDV rClone30s genome vector:

This experiment constructed NDVrClone30s by viral genome structure as shown in (Figure 6), pBrClone-P53-IL-2.

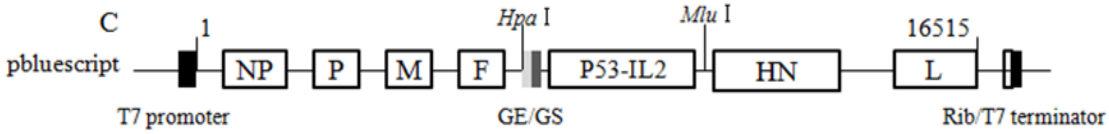

Figure (6): Tthe genes' structure of pBrClone30s
pBrClone30-P53-IL-2 gene.

Construction of rNDV-P53-IL-2 genome vector:

Positive recombinant plasmid PMD-18T-IL-2, was double digested by using a Mlu I and Sfi I endonuclease, 519bp fragment was recycled, ligated with prNDV-P53 large vector fragment, transformed with Escherichia coli Stbl2, single transformed colonies were picked, cultured in LB medium, then plasmid was extracted, identified by PCR using specific primers, Spe I restriction enzyme digestion was done at the same time with the identification of correct plasmid by PCR. Results are shown in (Figure 9), purpose of IL-2 could be obtained by PCR, after digestion, it indicates that the vector was constructed correctly; sequencing results also showed that the recombinant plasmid was successfully constructed.

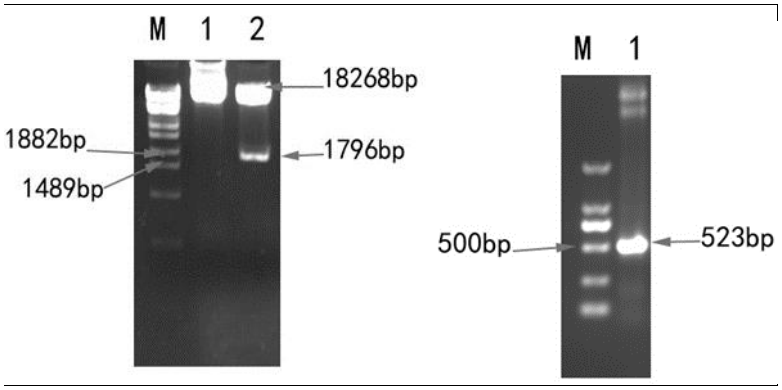

Figure (7): Identification of pBlrNDV-P53-IL-2 by PCR and enzyme digestion

(A) Lane M：λ-EcoT14 Lane1：pBlrNDV-P53-IL-2 plasmid Lane2：plasmid enzyme digestion product

(B) Lane M：DL2000Lane1: pBlrNDV-P53-IL-2 plasmid PCR identification product.

Rescue of pBlrNDV-P53-IL-2:

In order to get the infectious of Newcastle, Newcastle disease virus was constructed with new transcription of the full length cDNA plasmids pFLC-rNDV-P53-IL-2 and helper plasmid pTM1-N, pTM1-P, pTM1-L common-transfected BHK-21 cells. After transfection for 72h, transfected cell supernatants was collected, repeatedly freeze-thaw for 3 times at -80℃, inoculated to SPF 9-11-day-old chicken embryos, chicken eggs incubated at 37°C for 72h, allantoic fluid was collected under sterile conditions, hemagglutination (HA) test was done to determine the titer, take the positive results of allantoic fluid, passage 3 times, then the hemagglutination titer tests showed the titer=210, as shown in (Figure 8).

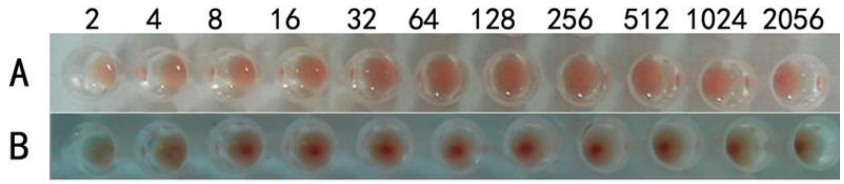

Figure (8): Hemagglutination（HA）test (A) control test (B) for rNDV-P53-IL-2.

Discussion:

While constructing recombinant NDV genome, nucleotide number meets the "six base principle" of the virus plays a decisive role in successfully rescuing the virus. NDV genome conserved sequence GS and GE are essential for viral transcription, added in the front and the end of the GS and GE sequences of the inserted foreign gene, and to ensure that the final completed constructed of recombinant NDVClone30s genome meet the "six-base principle"(12). In this study, BHK-21 cells were used for virus rescue; cells stably expressing the T7RNA polymerase, the viral rescue process conditions provide the NDV genome and the helper plasmid, negative-strand

RNA for transcription of RNA. In addition, we have selected NDV attenuated strain for virus rescue in our study, but the fusion precursor protein F0 of the attenuated strain could play a role in specific hydrolytic enzyme cleavage, since BHK-21 cells lack this enzyme, so for rescuing the virus in this study, we needed to add 1μg / ml trypsin to secondary assist rescuing of recombinant virus(13-15).

Gene sequencing results showed that the recombinant NDV genome vector constructed in this study are in line with the "six base principle" achieved the preconditions for virus rescue. HA experiments and experimental results of TCID50 recombinant NDV rescue in this study showed that the rescued recombinant NDV experimental are in line with the standards(16,17).

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