



Original Article

Oncolytic Activity of Wild-type Newcastle Disease Virus HK84 Against Hepatocellular Carcinoma Associated with Activation of Type I Interferon Signaling

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Abstract

Background and Aims: Hepatocellular carcinoma (HCC) is listed as one of the most common causes of cancer-related death. Oncolytic therapy has become a promising treatment because of novel immunotherapies and gene editing technology, but biosafety concerns remain the biggest limitation for clinical application. We studied the antitumor activity and biosafety of the wild-type Newcastle disease virus HK84 strain (NDV/HK84) and 10 other NDV strains. **Methods:** Cell proliferation and apoptosis were determined by cell counting Kit-8 and fluorescein isothiocyanate Annexin V apoptosis assays. Colony formation, wound healing, and a xenograft mouse model were used to evaluate *in vivo* and *in vitro* oncolytic effectiveness. The safety of NDV/HK84 was tested in nude mice by an *in vivo* luciferase imaging system. The replication kinetics of NDV/HK84 in normal tissues and tumors were evaluated by infectious-dose assays in eggs. RNA sequencing analysis was performed to explore NDV/HK84 activity and was validated by quantitative real-time PCR. **Results:** The cell counting Kit-8 assays of viability found that the oncolytic activity of the NDV strains differed with the multiplicity of infection (MOI). At an MOI of 20, the oncolytic activity of all NDV strains except the DK/JX/21358/08 strain was >80%. The oncolytic activities of the NDV/HK84 and DK/JX/8224/04 strains were >80% at both MOI=20 and MOI=2. Only NDV/HK84 had >80%

oncolytic activities at both MOI=20 and MOI=2. We chose NDV/HK84 as the candidate virus to test the oncolytic effect of NDV in HCC in the *in vitro* and *in vivo* experiments. NDV/HK84 killed human SK-HEP-1 HCC cells without affecting healthy cells. **Conclusions:** Intratumor infection with NDV/HK84 strains compared with vehicle controls or positive controls indicated that NDV/HK84 strain specifically inhibited HCC without affecting healthy mice. High-throughput RNA sequencing showed that the oncolytic activity of NDV/HK84 was dependent on the activation of type I interferon signaling.

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Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide.^{1,2} Although immunotherapy and targeted therapy have improved in recent years, global surveillance indicates a 5-year survival of 10–19% in most countries and regions.³ It is urgent to explore novel treatments for HCC. Oncolytic viruses have shown promise as a targeted HCC therapy,⁴ and as long ago as the 1950s and 1960s, it was reported that some viruses can eradicate cancer.^{5,6} The National Cancer Institute recommends virotherapy as a complementary cancer therapy.⁷ Over the last two decades, studies have focused on the modification of oncolytic viruses or the insertion of genes such as granulocyte colony stimulating factor (G-CFS) to enhance the cytotoxic effect on tumor cells, but no significant progress has been made.^{8,9} In addition, the safety of gene transfers, in which new DNA sequences are incorporated into the viral genomes to create “armed” oncolytic viruses has yet to be evaluated.

Some preclinical studies have evaluated oncolytic viruses in HCC. Adenoviruses have good tropism for hepatocytes,¹⁰

Keywords: Newcastle disease virus; Oncolytic effectiveness; Type I interferon; Hepatocellular carcinoma; Biosafety.

Abbreviations: CCK-8, cell counting Kit-8; CPE, cytopathic effect; DDP, cisplatin; EID50, egg infectious dose; GO, gene ontology; HCC, primary hepatocellular carcinoma; Hep3B, Hep3B human HCC cell lines; KEGG, Kyoto Encyclopedia of Genes and Genomes; MOI, multiplicity of infection; NDV, Newcastle disease virus; IFN, interferon; OV, oncolytic virus; qPCR, quantitative real-time PCR; RNA-seq, RNA sequencing; SK-HEP-1, SK-HEP-1 human HCC cell lines.

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Table 1. Inhibition of SK-HEP-1 cells by 10 NDV strains at different MOIs

Virus strain	Inhibition (%) at MOI=20	Inhibition (%) at MOI=2.0	Inhibition (%) at MOI=0.2
DK/JX/945/07	100.16	76.10	34.21
DK/JX/8224/04	98.59	89.69	28.89
WDK/JX/10487/04	98.49	67.36	27.41
DK/HK/2/1975	97.67	68.07	41.55
DK/JX/21332/08	86.34	60.55	36.84
DK/JX/18579/09	95.90	74.21	38.99
CK/ZZ/296/08	93.93	63.13	25.31
DK/HK/84/1976	88.90	86.12	83.19
DK/JX/21358/08	66.35	32.78	29.03
LaSota	84.29	41.26	10.53

NDV, newcastle disease virus; MOI, multiple of infection; DK, duck; JX, Jiangxi Province; WDK, wild duck; HK, Hong Kong; CK, chicken; ZZ, zhangzhou city.

vesicular stomatitis virus can infect tumor cells,¹¹ and vaccinia virus has been studied as a cancer virotherapy.^{12,13} Intratumor or intravenous administration of vaccinia virus JX-594, a modified Wyeth strain, was shown to inhibit the growth of malignant solid liver tumors in rodent models¹⁴ and to elimination of pulmonary metastases of hepatocellular carcinoma in rabbits.¹⁴ A few oncolytic viruses have been investigated in clinical studies. Adenovirus was reported to have no significant effect on HCC progression,¹⁵ and a study of vaccinia virus JX-594 (Pexa-Vec) was terminated early because the median overall survival did not reach the study endpoint.¹⁶ The common reasons for the termination of clinical trials were lack of specificity, lack of tropism, and transduction of tumor cells that resulted in ineffective clinical application, and adverse effects, including influenza-like symptoms, dose-related thrombocytopenia, and hyperbilirubinemia.¹⁷ Safety is a key concern that has restricted the development and selection of oncolytic virotherapy.¹⁸

Novel approaches for the virotherapy of solid tumors involve developing the potential of naturally oncolytic virus strains. The Newcastle disease virus (NDV) is a highly contagious poultry pathogen, but is nonpathogenic or mildly virulent in humans compared with vaccinia virus, herpes simplex virus or adenovirus.¹⁹ It has been studied for more than half a century,²⁰ and NDV receptors are widely expressed in humans.²¹ Various NDV strains have been evaluated for the treatment of tumors in preclinical studies, including the Hitchner B1 strain,²² the HUI strain,²³ the rNDV/F3aa strain,²⁴ the LaSota strain,²⁵ and the rAF-IL12 strain.²⁶ Clinical research is progressing slowly. Because the antitumor activity of NDV strains is associated with their phenotypes and molecular biological characteristics, research has focused on genetic modification or recombination of NDV strains to promote immunity²⁷⁻²⁹ and improve the tumor microenvironment³⁰ compared with the wild-type strain. Only a few NDVs have entered clinical trials, and none have been used in clinical practice. However, significant antitumor activity, selective replication in malignant cells, and low toxicity in human normal human cells^{20,31-33} have raised scientists' great stimulated interest in the oncolytic effects of NDV.

Potential NDV strains should have a low safety risk and high oncolytic effectiveness. Based on previous studies, we investigated the *in vivo* and *in vitro* oncolytic effectiveness and systemic safety of an NDV/HK84 strain identified within a group of 10 NDV strains. We confirmed its oncolytic effectiveness and low systemic toxicity, and RNA sequencing (RNA-seq) indicated that upregulation of genes regulating

the interferon (IFN) signaling pathway was involved.

Methods

Viruses and cell culture

The NDV LaSota strain was obtained from veterinarian vaccines and nine other NDVs were isolated from poultry in Southern China (Table 1). The viruses were stored at the Joint Institute of Virology (Shantou University and The University of Hong Kong), purified and grown in pathogen-free chicken eggs. All experiments were performed in biosafety level 3+ (BSL3+) laboratories. SK-HEP-1 and Hep3B human HCC cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in high-glucose Eagle's minimum essential medium (Gibco) containing 10% fetal bovine serum. The cells were incubated at 37°C in a 5% CO₂ humidified chamber.

Cell proliferation assay

Cell proliferation was assayed with a cell counting Kit-8 (CCK-8; AbMole, USA) following the manufacturer's instructions. SK-HEP-1 HCC cells were transferred to 96-well plates and incubated overnight before inoculation by NDVs and further incubation for specified time intervals. The infected cells were washed with phosphate buffered saline and then exposed to the CCK-8 reagents for 3 h. The effects of the NDV strains on cell proliferation were read at a wavelength of 450 nm with an iMark Microplate Reader (BIO-RAD Corp., USA). Inhibition of tumor-cell viability, i.e. cytotoxicity was reported as the percentage of living cells as previously described.³⁴ The assays were performed in triplicate. The effect of NDV/HK84 on Hep3B HCC cells was also determined by the CCK-8 assay. The effectiveness and safety of NDV/HK84 in normal liver cells was evaluated in AML12 normal mouse hepatocytes and PMH primary mouse hepatocytes by CCK-8 assays after overnight culture in 96-well plates.

Cytopathic effect (CPE) of NDV/HK84

NDV/HK84 or phosphate buffered saline (PBS) were added to SK-HEP-1 cells seeded in 96-well plates. The cultures

Table 2. The primer sequences of differentially expressed genes

Gene	Forward primer	Reverse primer
OASL	5'-GGAGTGGAAGGAAGAGGTGC-3'	5'-TTTCTCTGCAGCTCGCTGAA-3'
IFIT1	5'-CTAGCTCACTCCACGTAGCG-3'	5'-TGGTTTTGCCATTGCCAAGG-3'
ISG15	5'-TGCCAGTACAGGAGCTTG-3'	5'-ATTTCCGGCCCTTGATCCTG-3'
IFITM1	5'-AAACGACAGGGGAAAGGAGG-3'	5'-CAAAGTTGCAGGCTATGGG-3'
IRF7	5'-CAACCAAGGCTCCTGGAGAG-3'	5'-TACACCTTGCCTTGCAT-3'
DDX58	5'-CTGGTCCGTGGCTTTTGG-3'	5'-AGCAGGCAAAGCAAGCTCTA-3'
IFI44	5'-TTTGGAGGGAAGCGCTTAG-3'	5'-ATGCGTTACATGCCCTTGA-3'
MX1	5'-TCCGAAGTGGACATCGCAA-3'	5'-CAGCCACTCTGGTTATGCCA-3'

were incubated at 37°C and 5% CO₂, and CPE was assayed at 24, 48, and 72 h.

Apoptosis

SK-HEP-1 cells were seeded into 6-well plates and treated with NDV/HK84 (MOI = 2) or cisplatin (DDP; 7.5 µg/ml) for 24 or 48 h. The cells were harvested and washed in cold (PBS). Following the apoptosis detection kit manufacturer's (BD Pharmingen, USA) instructions, Annexin V FITC and propidium iodide were added to the cell suspensions for 15 min at room temperature in the dark. Binding buffer was added and apoptosis was assayed with a C6 flow cytometer and FowJo 10 (BD Pharmingen, USA) within 1 h.

Colony formation assay

SK-HEP-1 HCC cells (50 cells/well) or Hep3B HCC cells (100 cells/well) were seeded into a 6-well plate and cultured to 60% confluence. Cells were then treated with NDV/HK84 (MOI = 2), LaSota (MOI = 2) and DDP (7.5 µg/ml), or PBS for 48 h. The culture medium was replaced every 4–5 days with continuous culture for 2 weeks. Visible colonies, with at least 50 cells were stained with crystal violet, and counted by light microscopy.

Wound healing assay

SK-HEP-1 cells (2.0×10⁶ cells/well) were plated into 6-well plates and cultured to confluency. A 1 mm wide gap was scratched with a micropipette tip, detached cells were removed by washing with warm PBS. The attached cells were cultured with virus for specified times. The virus-containing medium was discarded, and the infected cells were washed twice with PBS. The wounds were observed and photographed with a phase-contrast microscope (ZEISS, DE) and the width of gaps in the monolayers were measured. The assays were performed in triplicate.

Cell invasiveness

Cell invasion was assayed in Matrigel coated Transwell chambers (Corning Inc., USA). SK-HEP-1 cells were treated with NDV/HK84, LaSota, DDP, or PBS for 48 h. Cells (1×10⁵) were suspended in 200 µL fetal bovine serum-free medium in the upper chambers of 12-well plates. The lower chamber was filled with 500 µL of medium containing 20% FBS to induce cell movement. After 24 h, the cells on the

lower surface of the membrane were fixed with 70% ethanol and stained with 0.5% crystal violet. The number of cells in five randomly selected fields (×100 magnification) were counted.

RNA sequencing (RNA-seq)

Total RNA was isolated with TRIzol (Takara Bio Inc., JPN). cDNA libraries were prepared by Illumina Paired End Sample Prep kits (Illumina Inc., USA) and sequenced with an Illumina HiSeq 4000 system (Illumina Inc., USA). Differential expression of transcripts in the treatment and control groups was measured. RNA-Seq was performed by Hangzhou Lianchuan Biotechnology Co., and differentially expressed genes were identified with the linear models for microarray data (limma) package in GEO2R; the cutoff criteria were *P* < 0.05 and a fold-change of >2.0.

Differential expression analysis and validation

A rigorous algorithm was used to identify differentially expressed genes. To determine significant of differences in gene expression, the threshold was a false discovery rate (FDR) ≤ 0.001 with an log₂ ratio absolute value ≥ 1. Cluster analysis of differentially expressed genes was with Cluster and Java TreeView. We annotated and mapped DEGs to terms in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases. DEGs were validated by quantitative real-time PCR (qRT-PCR). Primer sequences are shown in Table 2.

Xenograft mouse model

The procedures followed the guidelines of the animal experimentation ethics committee of Shantou University Medical College. BALB/c female nu/nu athymic nude mice (4–6 weeks old) were maintained in a pathogen-free environment. SK-HEP-1-Luc HCC cells (1×10⁷ cells/100 µL) were implanted by subcutaneous injection. Tumor volume was measured every 3 days with a digital caliper, and calculated as $V = 4/3 \times \pi \times S^2/2 \times L/2$, where S is the smallest diameter, V is the tumor volume and L is the largest diameter. When tumors were 5–7 mm in diameter, they were injected with 1×10⁷ egg infectious dose (EID₅₀) NDV/HK84 per 100 µL every 3 days (n=10 mice per group). There were five injections. PBS (100 µL) and 5 mg/kg DDP (100 µL) were controls. Body weight and behavior were monitored every other day; survival was monitored every day. Animals were sacrificed if (1) the 10% of the total body weight was lost

in 1 week; (2) the animal stopped feeding or drinking; (3) the tumors ruptured; (4) the tumors were >18 mm in any dimension (5) study termination had been reached. Mice were sacrificed by cervical dislocation when their condition was moribund.

Luciferase imaging

Before *in vivo* imaging, mice were anesthetized with isoflurane-oxygen and injected intraperitoneally with D-luciferin potassium salt 150 mg/kg (Xenogen, USA). An integration time of 6 m was used for acquisition of luminescent images with an *in vivo* imaging system (Perkin Elmer Corp., USA) and living image acquisition and analysis software (version 2.11; Xenogen Corp., USA).

Histopathology

Samples of the mouse tumors were obtained by dissection and fixed in 10% formaldehyde in pH 7.4 PBS. The fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin/eosin (H&E). The infiltration of immune cells was also observed.

Isolation and primary culture of mouse hepatocytes

The livers of 8-week-old wild-type male mice were perfused *in situ* via the postcava for 3 m with 42° calcium and magnesium-free HEPES buffer and for 5 m with CM HEPES buffer containing protease (14 mg/mouse) and for 7 m with collagenase D (3.7 U/mouse) at a flow rate of 3 mL/m. Isolated mouse primary hepatocytes were plated onto collagen-coated 6-well plates at a density of 2×10^5 cells/well. After the cells had attached for 6 h, the medium was changed to fresh medium supplemented with 10% FBS overnight.

Replication kinetics of NDV/HK84 in SK-HEP-1 and normal cells

We investigated the replication kinetics of NDV/HK84 in HCC and in normal tissue and evaluate its safety as previously described.²⁴ A dose of 1×10^7 NDV/HK84 cells in 100 μ L was injected into normal tissues in the left flank or tumors in the right flank of nude mice. The mice were killed at 1, 2, 3, 4, 5, 6, 7, or 8 days after treatment and pieces of normal and tumor tissue were collected and prepared for determination of the median viral embryo infective dose (EID₅₀).

Statistical analysis

The values of continuous variables were reported as means \pm standard deviation (SD). Multigroup comparisons were performed by one-way analysis of variance followed by Dunnett's test. Welch's analysis of variance followed by Dunnett's T3 test was used for data with unequal variances. Differences in the values that were not normally distributed were compared by Kruskal-Wallis tests; between-group differences were compared by Mann-Whitney U tests. $P < 0.05$ was considered statistically significant. The statistical analysis was performed with SPSS 23.0 (IBM Corp., Armonk, NY, USA) and Graph Pad Prism 8.0 (Graph Pad software, La Jolla, CA, USA).

Results

Inhibition and cytopathic effect (CPE) of NDVs on SK-HEP-1 HCC cells were high regardless of MOI

Inhibition of SK-HEP-1 HCC cells by the 10 NDV strains is shown in Figure 1A. The oncolytic activity of the NDV strains was >80%, except DK/JX/21358/08. The oncolytic activities of NDV/HK84 and DK/JX/8224/04 were more than 80% at MOIs of 20 and 2. As only NDV/HK84 had more than 80% inhibition at all three MOIs was chosen as a novel NDV strain for further evaluation. The CCK-8 assay results showed significant inhibition of the proliferation of SK-HEP-1 cells with increasing concentrations of NDV/HK84 for 72 h. At all three MOI values, the inhibition of SK-HEP-1 cells was >80% (Fig. 1A). Even at a low concentration (MOI=0.2), the inhibition rate was high (Fig. 1B, D). The median effective concentration (EC₅₀) of NDV/HK84 was 0.0019 MOI for SK-HEP-1 (Fig. 1C) and was 0.6159 for Hep3B, which demonstrated good antitumor activity (Fig. 1E). The CPE on HCC cells at 24, 48, and 72 h post-inoculation included rounding, detachment from the culture surface, and death (Fig. 1F, G).

NDV/HK84 induced apoptosis and inhibited HCC cells proliferation

Apoptosis plays a key role in both tumor development and treatment. Flow cytometry showed that after treatment for 48 h, NDV/HK84 caused a significant increase in late apoptosis (right upper quadrant) compared with either the PBS vehicle or positive DDP control (Fig. 2A). The percentages of late apoptotic cells were 5.41% for PBS, 31.20% for DDP, and 67.27% for NDV/HK84 ($p < 0.05$, Fig. 2A). In the colony formation assay, the anti-proliferation activity of NDV/HK84 resulted in a time-dependent decrease of colony formation by both SK-HEP-1 and Hep3B HCC (Fig. 2B, C). The inhibition of proliferation by NDV/HK84 was greater than that by LaSota (Fig. 2B, C).

NDV/HK84 suppressed *in vitro* migration and invasion of hepatocellular carcinoma cells

The wound healing assay (Fig. 2D) showed that NDV/HK84 caused a time-dependent reduction in cell migration compared with the control. DDP and the LaSota NDV also inhibited cell migration, but the most significant decrease of migration distance at each time of measurement was found in the NDV/HK84 group (Fig. 2D). In the Transwell assays, at each concentration that was tested, significantly fewer SK-HEP-1 cells in the upper chambers, and the numbers of migrating to the lower membrane surface in the NDV/HK84 group than in the DDP and LaSota groups (Fig. 2E). Taken together, the results demonstrate that NDV/HK84 inhibited HCC cell migration and invasiveness.

NDV/HK84 inhibited tumor growth in nude mice

Subcutaneous injection of SK-HEP-1 cells into the right hip flank of nude mice was followed by the development of visible tumors within 7 days. When the tumors were 5–7 mm in diameter, they were injected with a 100 μ L suspension containing 1×10^7 EID₅₀ of NDV/HK84 every 3 d for a total of five injections. PBS and DDP (5 mg/kg) were negative and positive controls (Fig. 3A). Tumor inhibition was stronger with NDV/HK84 than with PBS and DDP. In six of 10 mice

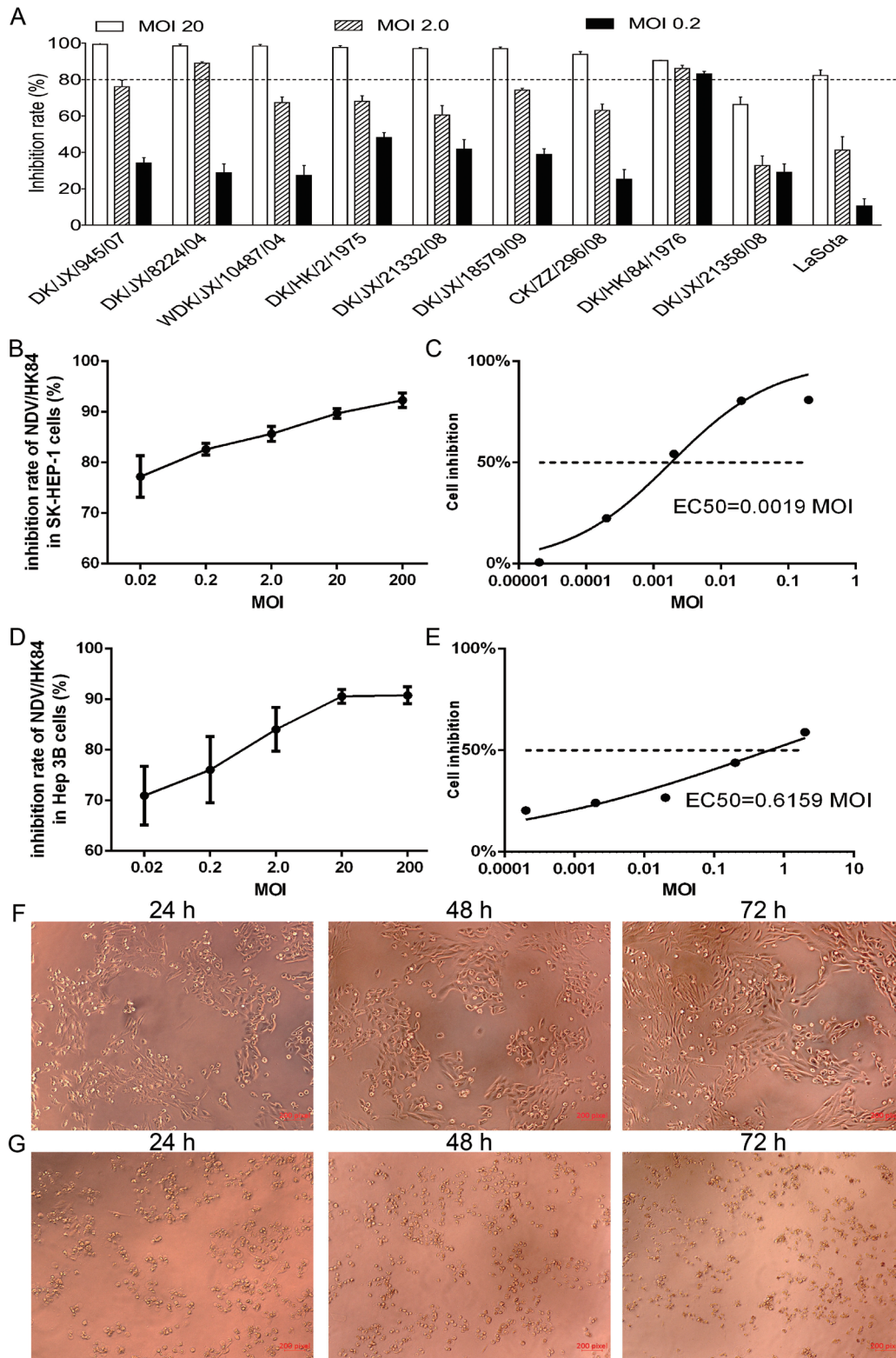


Fig. 1. Inhibition, EC₅₀ and CPE of NDV/HK84 on HCC cells. (A) Inhibition rates of 10 NDV strains on SK-HEP-1 cells. (B) At different concentrations, NDV/HK84 significantly inhibited the proliferation of SK-Hep-1 cells. (C) The EC₅₀ of NDV/HK84 on SK-HEP-1 cells. (D) NDV/HK84 inhibition of Hep3B cells at different MOIs. (E) The EC₅₀ of NDV/HK84 on Hep3B cells. (F) CPE of the negative control (PBS) on SK-HEP-1 cells at different times post-inoculation. (G) CPE of NDV/HK84 on SK-HEP-1 cells at different times post-inoculation. EC₅₀, the median effective concentration; CPE, cytopathic effect; NDV/HK84, newcastle disease virus HK84; HCC, primary hepatocellular carcinoma; MOI, multiplicity of infection.

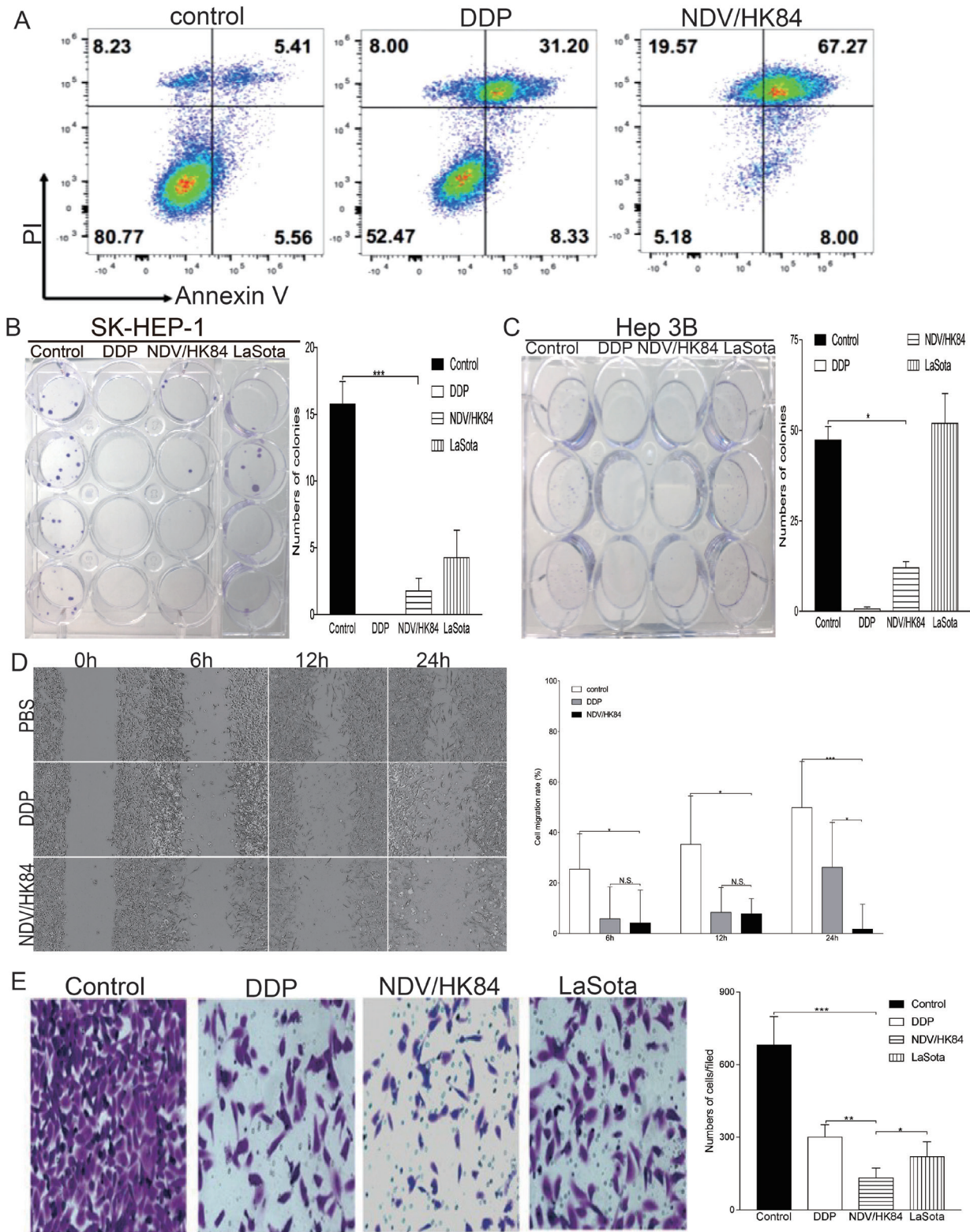


Fig. 2. NDV/HK84 induced apoptosis, and inhibited proliferation, migration, and invasiveness on HCC cells. (A) Apoptosis was assayed by flow cytometry after staining with Annexin V and PI, and treatment by PBS control, DDP (7.5 $\mu\text{g}/\text{ml}$), or NDV/HK84 (MOI=2) for 48 h. (B, C) NDV/HK84 and DDP significantly inhibited colony formation compared with the LaSota strain and PBS. (D) Wound-healing assays showed that NDV/HK84 significantly inhibited migration at 24 h compared with controls. (E) Transwell assays (representative images, $\times 200$ magnification) showed that NDV/HK84 significantly inhibited cell migration compared with PBS, DDP, or the LaSota strain. Data are means \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PBS, phosphate buffered solution; DDP, cisplatin.

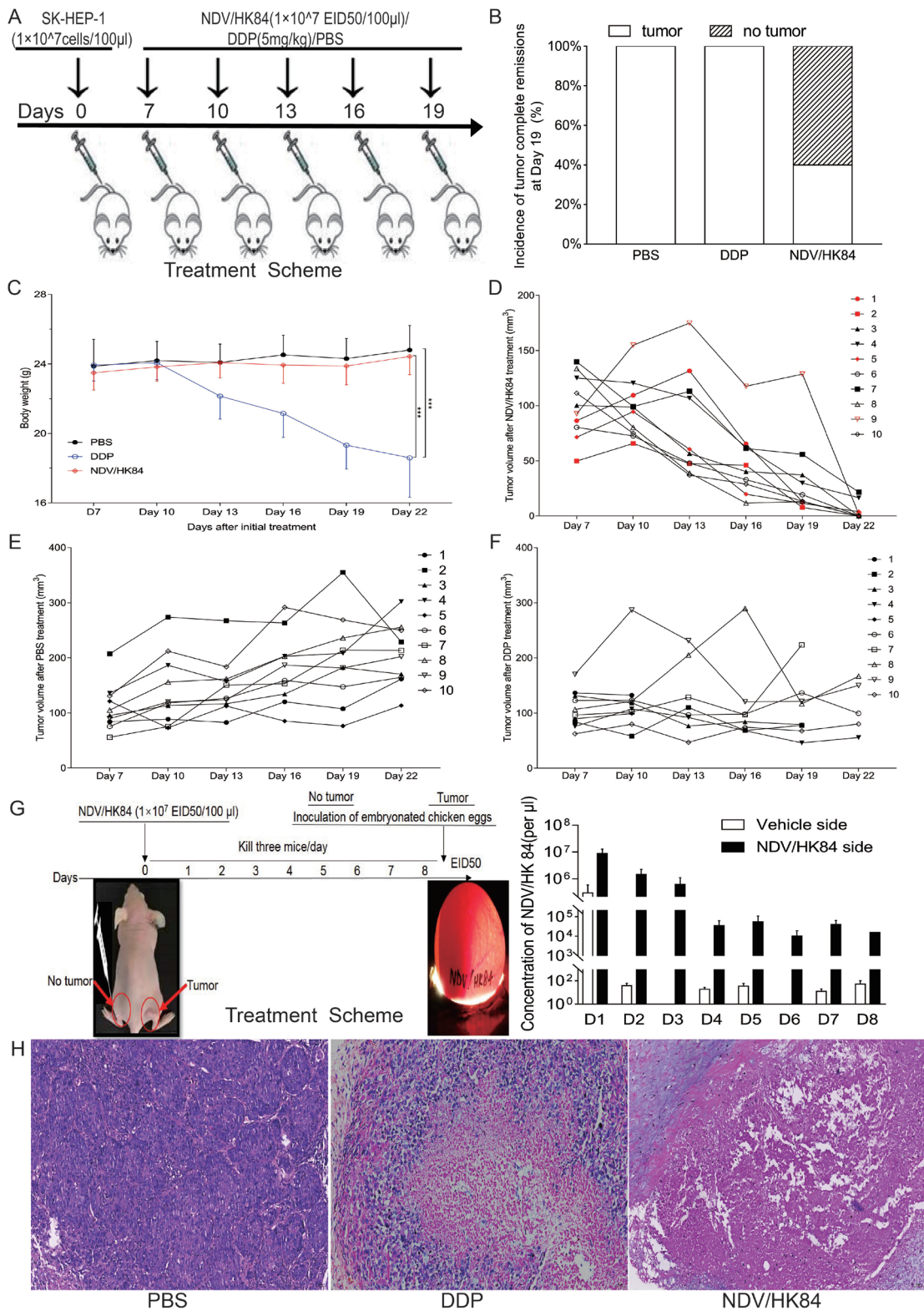


Fig. 3. NDV/HK84 inhibits tumorigenicity in the xenograft model. (A) Mice were subcutaneously injected with SK-HEP-1 cells and treated with PBS, DDP, or NDV/HK84, on days 7, 10, 13, 16 and 19. (B) Mice with complete tumor remission. (C) Body weight of DDP-, NDV/HK84-, or PBS-treated mice. Data are means \pm SD. (D) Tumor volume in NDV/HK84-treated mice. (E) Tumor volume in PBS-treated mice. (F) Tumor volume DDP-treated mice. (G) NDV/HK84 viral titers in normal tissues and tumors after infection by EID₅₀. Data are means \pm SD. (H) Representative sections of hematoxylin and eosin-stained tumor tissues collected on day 15 the first injection of NDV/HK84, DDP, or PBS. EID₅₀, 50% egg infective dose.

treated with NDV/HK84, the tumors completely regressed after 2 weeks of treatment (Fig. 3B). On day 19, 2 weeks after the first injection, the average tumor volumes were 206.3 mm³ in the control group, 110.6 mm³ in the DDP group, and 4.3 mm³ in the NDV/HK84 group. The growth in tumor volume was significantly suppressed by NDV/HK84, and the difference in tumor volume in the NDV/HK84 and DDP groups was significant (Fig. 3D–F). The results are evidence of the antitumor activity of NDV/HK84.

Histopathological evaluation of hematoxylin and eosin stained tissue revealed tumor necrosis in tumors treated with DDP and NDV/HK84. Few viable tumor cells were visible, but large patches of necrosis with some scattered apoptotic or tumor cells were present (Fig. 3H).

Safety evaluation of NDV/HK84 strain

Decreases in *in vivo* luciferase expression in tumor cells occurred along with decreases in tumor volume after treatment with DDP positive control and NDVs viruses during the 15 day study period (Fig. 4A–F). Six of 10 subcutaneous tumors in the NDV/HK84 group were no longer measurable (Figs. 3D, 4F). Mice in the DDP group, the nude mice developed significant weakness and weight loss (Figs. 3C, 4E), and experienced less tumor shrinkage than mice the NDV/HK84 group (Figs. 3F, 4E). Three mice in the DDP group lost more than 20% of the body weight and were sacrificed for ethical reasons. Mice in the NDV/HK84 group, did not experience any obvious therapy-associated side effects or weight loss (Fig. 3C). The results indicate that NDV/HK84 had a better safety profile than DDP.

The NDV/HK84 EID₅₀ results demonstrated tumor-specific viral replication and little replication in normal cells. In the 8 days after treatment, the viral titer of tumors in nude mice decreased, but remained at a relatively high level. In normal tissues, the viral titer rapidly dropped to near 0 on day 2 of treatment (Fig. 3G). This persisted until the eighth day of the experiment, showing that NDV/HK84 replicated poorly in normal tissues and had good safety. CCK-8 assays in normal AML12 mouse hepatocytes AML12 and PMHs found that at a low MOI (0.02), NDV/HK84 had nearly no effect on proliferation or the viability normal liver cells (nearly 0%), which was significantly different from the effects on HCC cells. As MOI increased, the inhibition of normal hepatocytes by NDV/HK84 remained low. The results show that NDV/HK84 killed tumor cells selectively (Fig. 4G, H).

Global changes in mRNA expression in SK-HEP-1 HCC cells after NDV/HK84 infection

The results of RNA-seq analysis of NDV/HK84 treated SK-HEP-1 cells and the upregulated and the downregulated genes are summarized in Figure 5A and differences in transcript abundance are shown in a Venn diagram (Fig. 5B). GO enrichment analysis found significant enrichment in type I Interferon signaling and innate immune-response genes in control and NDV/HK84 groups (Fig. 5C). KEGG enrichment analysis found that retinoic acid inducible gene (RIG)-1-like receptors and Toll-like receptors were involved in NDV/HK84 treatment (Fig. 5D).

Upregulation of type I interferon signaling in SK-HEP-1 cells following NDV/HK84 infection

GO and KEGG enrichment analysis found that some immune reaction-related genes participated in the events triggered

by NDV/HK84 infection in SK-HEP-1 cells. Human oligoadenylate synthetase-like (*OASL*),^{35,36} XIAP-associated factor 1 (*XAF1*),^{37–39} IFN-stimulated gene15 (*ISG15*),^{40,41} interferon-induced transmembrane protein 1 (*IFITM1*),^{42,43} MX dynamin like GTPase 1 (*MX1*),^{44,45} interferon regulatory factor 7 (*IFR7*),^{46,47} interferon-induced protein 44 (*IFI44*),⁴⁸ and DExD/H-box helicase 58 (*DDX58*) genes,^{49,50} and those associated with the interferon signaling pathway were manually selected (Table 3). The RNA-seq analysis results were validated by real-time PCR (Fig. 5E). In addition, lymphocyte, neutrophil, macrophage, fibroblast, and plasma cell infiltration was observed in the tumor tissue xenografts in nude mice treated by NDV/HK84 but not in the control group (Fig. 5F). The results are consistent with the involvement of type I IFN signaling in the inhibition of HCC by NDV/HK84 (Fig. 5G).

Discussion

HCC has high morbidity and mortality. Poor survival highlights the need for new drugs. NDV is cytotoxic to many cancer cell lines with diverse embryonic origin,⁵¹ including nervous, connective and epithelial tissues. It is not cytotoxic to normal tissues.²⁰ Many *in vitro* and *in vivo* studies have reported the oncolytic effectiveness and good safety profiles of NDVs,²⁰ but the progress of NDV-dependent oncolytic therapy for HCC has been slow. Genetic engineering and gene editing may be able to increase the oncolytic effectiveness of NDVs,^{22,23,26} but the roles of specific phenotypes and the molecular mechanisms of NDV oncolysis remain poorly understood.²⁰ The potential biosafety of genetically engineered NDV strains have been intentionally or unintentionally ignored. There have still not been any successful clinical studies of NDVs for HCC treatment. Hence, the exploration of novel wild-type NDVs with high oncolytic effectiveness is urgent and deeply meaningful.

We have previously reported on the collection of dozens of NDVs for avian influenza and other virus research. The oncolytic effectiveness and safety of NDV in tumor therapy has been reported.²⁰ our intention was to find novel natural novel NDV strains with low risk and high oncolytic effectiveness. The hope is that systematic screening of the existing wild-type NDV- strain pool would offer a new perspective for HCC oncolytic therapy.

NDV strains have differing tumor inhibition activity.^{22–26} Pap *et al.* showed that MTH-68/H was cytotoxic to 13 human melanoma cell lines, and that but their EC₅₀ values were significantly different.⁵³ Kseniya S. Yurchenko *et al.* reported high oncolytic activity of seven of 44 natural pigeon NDV strains against diverse cancer-cell lines.⁵⁴ In this study, even for the same SK-HEP-1 liver cancer cell line, the tumor inhibition rates of different NDV strains varied significantly. We choose the NDV/HK84 strain to test its potential oncolytic effectiveness and safety in HCC cell lines and in nude mouse xenografts because of its inhibition rate of 86.12%.

Compared with a DDP positive control and the NDV LaSota strain, NDV/HK84 significantly inhibited the proliferation, migration, and invasiveness of SK-HEP-1 cells and the cytotoxic effectiveness of NDV/HK84 on HCC was better than that of cisplatin. In addition, the CPE of NDV/HK84 on SK-HEP-1 cells was significant. We observed the cytotoxicity of NDV/HK84 within 24 h, and within 72 h, the cells became round, necrotic, exfoliated, formed clusters, and died. We also investigated the oncolytic effectiveness of NDV/HK84 in HCC in xenotransplants in nude mice. NDV/HK84 inhibition of tumor development was superior to that of DDP. Fifteen days after the first intratumoral injection of NDV/HK84, subcutaneous tumors in the right flank of six of 10 nude

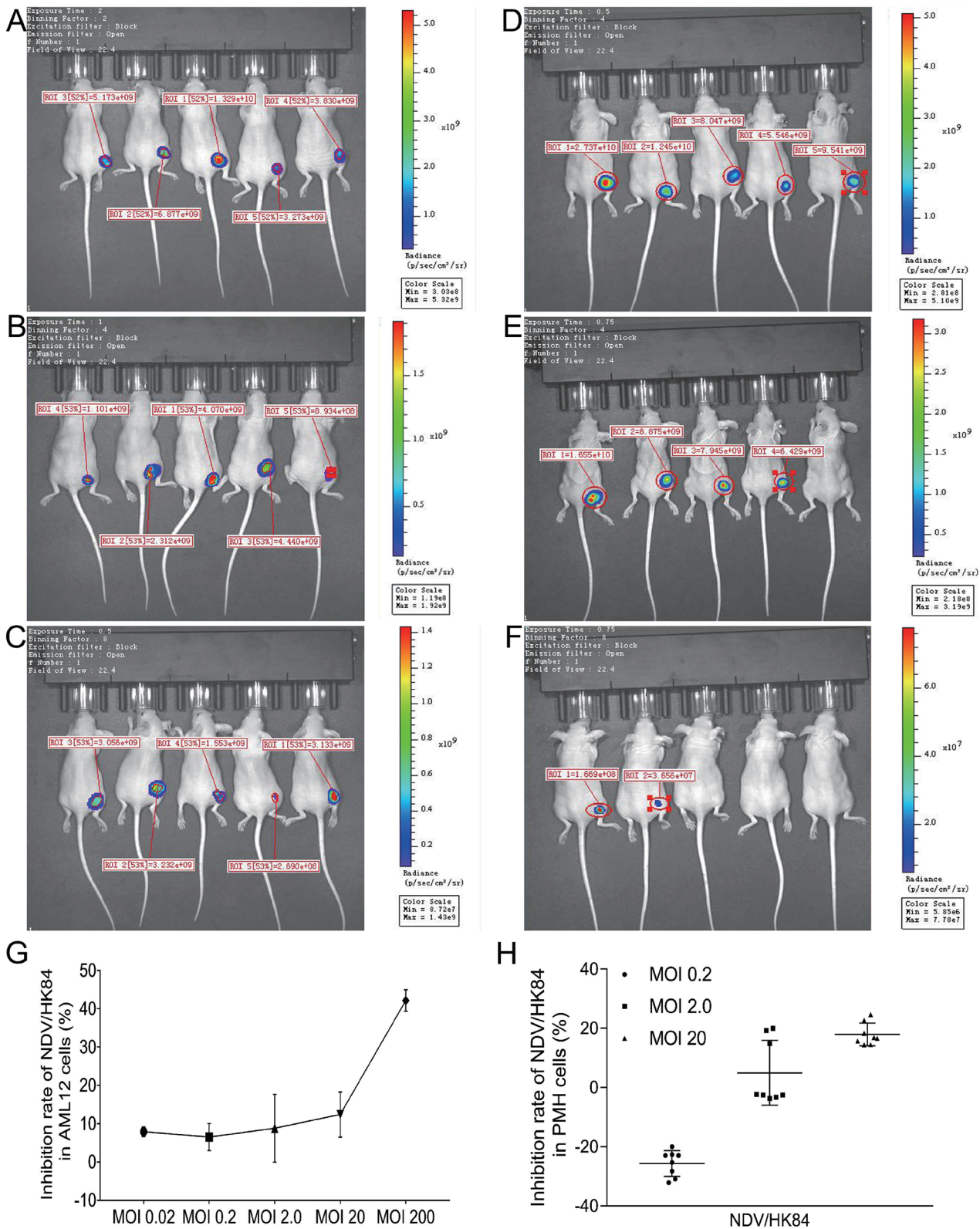


Fig. 4. Imaging of luciferase expression in nude mice after the first and fifth injections during the 15 day study period. (A–C) Mice injected with PBS (A), DDP (B), or NDV/HK84 (C) after the first injection. (D–F) Mice injected with PBS (D), DDP (E), or NDV/HK84 (F) after the fifth injection. Compared with the first injection, fluorescence intensities in two nude mice decreased (F). No fluorescence was detected in three mice, indicated the tumors had vanished after NDV/HK84 treatment. (G, H) Inhibitory rates of NDV/HK84 in normal mouse hepatocytes, AML12 cells, and primary mouse hepatocytes At MOIs of 0.02, 2 and 20.

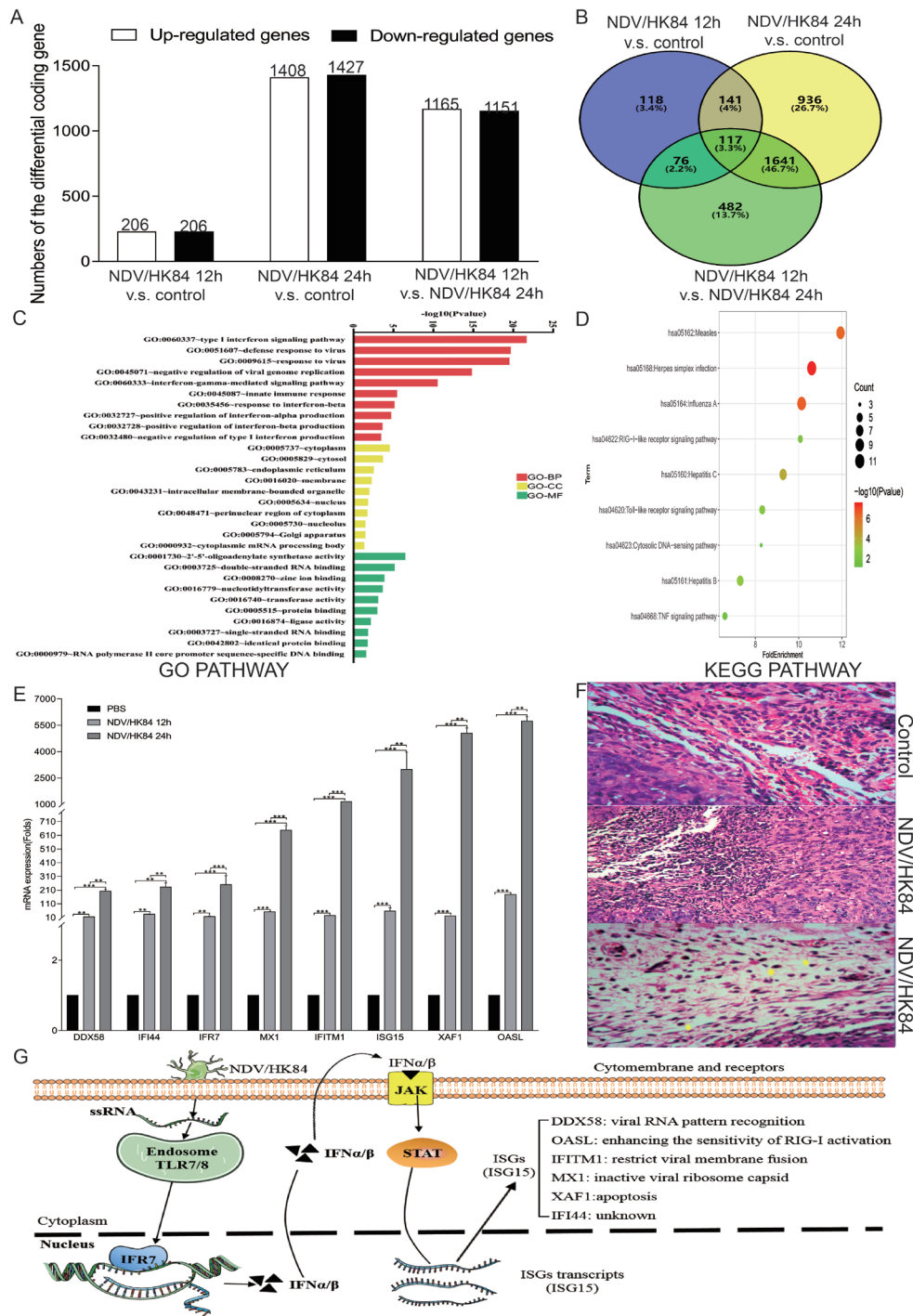


Fig. 5. IFN signaling pathway in SK-HEP-1 cells after NDV/HK84 treatment. (A) RNA-seq and analysis of differential gene expression in SK-HEP-1 cells treated for 12 h and 24 h by bioinformatics tools. White indicates upregulated genes and black indicates downregulated genes. (B) Venn diagram showing differences in transcript abundance. (C, D) GO and KEGG enrichment analysis of the putative functions of differentially expressed genes. (E) Transcription of *OASL*, *XAF1*, *ISG15*, *IFITM1*, *MX1*, *IFR7*, *IFI44*, and *DDX58* genes assayed by qRT-PCR in SK-HEP-1 cells treated by NDV/HK84 for 12 h and 24 h. (F) Increased infiltration of immune cells in the xenograft model following NDV/HK84 treatment. Representative hematoxylin and eosin staining of tumor tissue from control mice (top, without immune infiltration, $\times 200$), and NDV/HK84-treated mice (middle, showing extensive inflammatory cells, $\times 200$; bottom infiltration of immune cells in the xenograft model following NDV/HK84 treatment. Representative hematoxylin and eosin staining of tumor tissue from control mice (top, without immune infiltration, $\times 200$), and NDV/HK84-treated mice (middle, showing extensive inflammatory cells, $\times 200$; bottom infiltration of lymphocytes, neutrophils, macrophages, fibroblasts, and plasma cells, $\times 400$). (G) Diagram of the potential oncolytic mechanism of NDV/HK84 by activating type I IFN (IFN α/β) signaling against hepatocellular carcinoma. After invading liver cancer cells, NDV/HK84 is recognized by intracellular innate immune receptors to activate an immune response mediated by type I IFN. Many interferon-stimulating genes, including *DDX58*, *OASL*, *IFITM1*, *MX1*, *XAF1*, *IFI44* and *ISG15* are involved in the initiation of antitumor immunity. IFN, interferon; RNA-seq, RNA Sequencing; GO, Gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; qPCR, real-time quantitative PCR; OASL, human oligoadenylate synthetases-like; XAF1, XIAP-associated factor1; ISG15, IFN-stimulated gene15; IFITM1, interferon-induced transmembrane protein1; MX1, MX dynamin like GTPase 1; IFR7, IFN transcription factor; IFI44, interferon-induced protein 44; DDX58, DExD/H-box helicase 58 (RIG-1).

Table 3. Differentially expressed genes and their functions in HCC cells treated with NDV/HK84

Gene symbol	Gene name	Location	Function	Relationship to cancer	Ref.
OASL	human oligoadenylate synthetases-like	12q24.31	Promotes antiviral activity by enhancing the sensitivity of RIG-I activation	Association with immune cell infiltration in pancreatic cancer ³⁶	35,36
XAF1	XIAP-associated factor1	17p13.1	unknown	Promoted apoptosis either by p53 stabilization, ³⁷ or control of G2/M phase, ³⁸ induced autophagy by upregulating Beclin 1, or inhibiting AKT signaling ³⁹	37–39
ISG15	IFN-stimulated gene15	1p36.33	An interferon-induced protein that has been implicated as a central player in the host antiviral response	Direct impact on the pleiotropic cellular functions of ubiquitin, and leading to several human diseases including cancer ⁴¹	40,41
IFITM1	interferon-induced transmembrane protein1	11p15.5	Restrict viral membrane fusion	Knock down of IFITM1 regulated the proliferation, cell cycle arrest and apoptosis, and disturbed the MAPK signaling ⁴³	42,43
MX1	MX dynamin like GTPase 1	21q22.3	Inactivate viral ribosome capsid through Toll-like receptor signaling pathway to prevent viral genome transcription	unknown	44,45
IFR7	IFN transcription factor	no	Acts within the JAK/STAT pathway in response to viral infection by regulating IFN	Antitumor effects through inducing TNF-related apoptosis signaling ⁴⁷	46,47
IFI44	interferon-induced protein 44	1p31.1	unknown	Anti-proliferative activity in melanoma cell ⁴⁸	48
DDX58	DExD/H-box helicase 58 (RIG-1)	9p21.1	An intracellular “whistler”, an important pattern recognition receptor (PRR) for viral RNA. Induction of IFN and proinflammatory cytokines	Suppresses the migration and invasion of HCC ⁵⁰	49,50

HCC, primary hepatocellular carcinoma; IFN,interferon; OASL, human oligoadenylate synthetases-like; RIG-I, Ret inoic acid inducible gene I; XAF1, XIAP-associated factor1; G2/M, G2 and M cell cycle; ISG15, IFN-stimulated gene15; IFITM1, interferon-induced transmembrane protein1; MX1, MX dynamin like GTPase 1; IFR7, IFN transcription factor; IFI44, interferon-induced protein 44; DDX58, DExD/H-box helicase 58 (RIG-1).

mice completely disappeared, with no visible measurable lesions. Hematoxylin and eosin staining of tumors from NDV/HK84 group mice with tumor regression revealed almost no viable tumor cells. We were surprised to find complete tumor regression in six of 10 tumors treated with a wild-type NDV/HK84 strain. Vigil *et al*. previously reported complete regression of two of 10 tumors in a rat model treated by a genetically engineered NDV (rNDV/F3aa). Complete regression was not observed in 10 tumors treated by wild-type NDV/B1.²² In addition, in this study, the NDV/HK84 infected mice gained weight and had good vitality, compared with the vehicle and DDP groups. The NDV/HK84 strain had excellent oncolytic-effectiveness and safety for HCC treatment, which need confirmation in other tumor types.

The study focus was to verify the oncolytic ability of NDV/HK84. We had great interest in its oncolytic mechanism because of its excellent effectiveness in animal experiments. Differential gene expression profiles were significantly enriched in genes involved in type I IFN signaling and innate immune responses. Type I IFN is a powerful antiviral agent that rapidly induces transcription of interferon-stimulated genes, and is involved in the first-line antiviral defense after pathogen invasion, especially viral infection.⁴⁰ The induced products provoke antiviral effects by activating Janus ty-

rosine kinase-signal transducer and activator of transcription (JAK-STAT) signaling.⁵⁵ Type I IFN is also involved in the innate immune response to developing malignancies by actively participating in cancer immunosurveillance. It remains to be determined which tumor-derived products and signal transduction pathways underlie such an effect.⁵⁶ Therefore, based on our results, we speculate that NDV/HK84 induces antitumor immunity through the type I IFN pathway. As we expected, RNA-seq analysis found that RIG-I like receptors, Toll-like receptors, and type I IFN signaling were significantly enriched. Eight differentially expressed genes related to the IFN pathway were chosen to validate the fidelity of RNA-seq. Because the understanding of the IFN-stimulated genes is limited, it is essential to describe the nature of the host increase linked to type I IFN signaling and oncolytic effectiveness associated with NDV/HK84 infection. The roles of some of the eight candidate genes have been described. Oligoadenylate synthase-like (OASL) protein increases the RIG-I activity to modulate immune response.³⁵ Interferon-stimulated gene 15 (*ISG15*) is rapidly upregulated after stimulation by IFN, and *ISG15* knockout mice increased susceptibility to influenza, herpes, and Sindbis virus infection.⁴⁰ An IFN-stimulated gene (ISG) protein family member, interferon-induced transmembrane protein

1 (IFITM1) participates in the prevention of viral entry into host cellular membrane at the early stage of virus infection.⁴² IFITM1 has antiproliferative activity and participates in immune surveillance and tumor suppression.⁴³ MX1 encoding protein (MX dynamin like GTPase 1) generates a protective antiviral response by sensing nucleocapsid-like structures under increased IFN- α stimulation by Toll-like receptors signaling.^{44,45} DExD/H-Box Helicase 58 (DDX58) is a pattern recognition receptor for viral RNA in sensing viral nucleic acids and an intracellular signaling protein in maintaining innate immune system homeostasis.⁴⁹ We failed to find any strong correlations or associations of the eight candidate genes with liver tumorigenesis or host immune response induced by a virus. Lymphocytes and other immune cells were observed in the tumor tissue that was treated with NDV/HK84. The result indicated that NDV/HK84 may trigger the IFN pathway, recruit inflammatory factors, and kill tumor cells.

Although the candidate genes are involved in the innate immune response to virus infection, it remains unknown whether the eight candidate genes are principal components or signals directly responsible for the oncolytic ability of NDV/HK84. Our results provide evidence that the oncolytic effectiveness against HCC was linked to the activity of type I IFN triggered by infection by the wild-type NDV/HK84 strain. The molecular mechanism that links the candidate genes with the oncolysis or second bystander killing by NDV/HK84 and the canonical innate antiviral activity need investigation.

In summary, the wild-type NDV/HK84 strain had a strong oncolytic effect against HCC cells and was safe in a nude mouse model. The RNA-seq results were consistent with upregulation of interferon signaling as the major oncolysis-related pathway. The IFN-related genes were very important elements in the inhibition of SK-HEP-1 cells by NDV/HK84. Future studies should focus on the molecular biological characteristics of the NDV strains and the oncolytic molecular mechanism of NDV/HK84. We believe that this novel wild-type NDV/HK84 strain will be useful in future clinical trials of oncolytic therapies for HCC and other tumor species.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Drafting manuscript (LC), data collection (MX, JS, DS), sta-

tistical analysis (HL), and modification of the manuscript for intellectual content (YG, YN). All authors read and approved the final version of the manuscript.

Data sharing statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. The NDV virus strains of this study are available from the corresponding author, upon reasonable request.

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