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Review Article

Safety and Clinical Usage of Newcastle Disease Virus in Cancer Therapy

Han Yuen Lam,[1] Swee Keong Yeap,[2] Mehdi Rasoli,[2] Abdul Rahman Omar,[2] Khatijah Yusoff,[3] Abd Aziz Suraini,[4] and Noorjahan Banu Alitheen[1]

[1] *Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2] *Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3] *Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[4] *Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

Correspondence should be addressed to Noorjahan Banu Alitheen, noorjahan@biotech.upm.edu.my

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Newcastle disease virus (NDV) is an avian virus that causes deadly infection to over 250 species of birds, including domestic and wild-type, thus resulting in substantial losses to the poultry industry worldwide. Many reports have demonstrated the oncolytic effect 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 favorable 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. This paper provides an overview of the current studies on the cytotoxic and anticancer effect of NDV via direct oncolysis effects or immune stimulation. Safety of NDV strains applied for cancer immunotherapy is also discussed in this paper.

1. Introduction

Cancer is a life-threatening disease characterized by uncontrolled cell division leading to invasion of surrounding tissues and metastasis. Cancers arise from both genetic and environmental factors that lead to aberrant growth regulation of stem cell populations, or by the dedifferentiation of more mature cell types. Despite modern advance techniques in diagnosis, prevention, and therapy, cancer is still affecting millions of patients worldwide and causing high mortality [1].

In fact, cancer is a leading cause of death worldwide which accounted for 7.9 million deaths (around 13% of all deaths) in 2007 [2]. According to the report from the National Cancer Registry Malaysia [3], the age-standardised incidence rate (ASR) for all cancers in year 2006 was 131.3 per 100,000 people, regardless of sex and age. The five most common cancers among the population of Peninsular Malaysia in 2006 were breast, colorectal, lung, cervix, and nasopharynx cancers.

The ideal cancer therapeutic is based on the selectively killing of the malignant cells, while leaving normal tissues intact. Currently, radiotherapy, chemotherapy, and surgery are the most common treatments in cancer therapy. However, these therapies frequently lead to deleterious severe side effects [4]. Hence, it is important to develop a cancer therapy with high efficacy selectivity killing malignant cells with fewer pitfalls. Virotherapy using oncolytic viruses had been proposed as a potent cancer therapeutic. However, the application of viruses in cancer therapy is still under review. Thus, the focus of this paper relates to the safety and

preclininal/clinical experiences of utilizing NDV strains in cancer treatment.

2. Virotherapy

Seventy years ago, a lot of viruses have been discovered to carry oncolytic activity against tumor cells. These viruses include adenovirus, rabies virus, poliovirus, herpes simplex virus, hepatitis A virus, influenza A virus, measles virus, and NDV. Viruses can be genetically engineered to enhance their cytolytic abilities. For example, recombinant oncolytic herpes simplex virus that expresses DF3/MUC1 antigen is replicated preferential in colon cancer liver metastasis, rather than normal liver cells [5]. Several viruses are genetic manipulated to specifically target the cancer cells. Introducing ONYX-015 (dl1520), a replication-selective adenovirus, which had been modified by the deletion of the E1B-55-kd region, enables the p53 proteins to maintain their functions [6]. Therefore, the virus replication is dependent on the expression of the p53 proteins. Thus, the virus replicating is inhibited in cells with normal p53 function; in contrast, malfunction of p53 proteins in tumor cells may lead to replication and cell killing. In some cases, the virus is applied in such a way that the virus attenuates in normal cells, without affecting its cytolytic ability towards tumor cells.

3. Immunotherapy

Immunotherapy refers to a new form of treatment strategies which modulate the immune system to achieve a therapeutic goal, including cancer treatment. Cancer immunotherapy began in the late 1800s, where William Coley prepared a mixed vaccine of *streptococcal* and *staphylococcal* bacteria, known as Coley's toxin, which helped to control or even cure a few advanced cancers [7]. An immunomodulator agent has the ability to augment immune defenses and treat immunodeficiencies, cancer, infections and even autoimmune disorders [8]. One example is the introduction of the tuberculosis vaccine, Bacillus Calmette-Guerin (BCG) that can help to stimulate the immune system and eradicate lung carcinoma [9].

Immunotherapy exploits the properties of the immune system, which involves the white blood cells (WBC), such as natural killer (NK) cells, and T and B lymphocytes. The B lymphocytes produce antibodies targeting foreign antigens. The T lymphocytes are activated by other cells, as well as secrete cytokines useful for cell activation, proliferation, and differentiation, in response to specific invader antigens. NK cells are activated by the cytokines, in response to tumor cells and pathogens. Activated NK cells also secrete cytokines such as interferon (IFN), interleukin (IL), tumor necrosis factor (TNF), and others [10, 11].

4. Newcastle Disease Virus

Newcastle disease (ND) was the name given to a highly pathogenic disease when occurred in England by Alexander [12]. This disease has plagued the poultry industry since it was first recognized in 1926. It was caused by NDV, a virus category in the family *Paramyxoviridae* and genus *Avulavirus* [13]. NDV is also named as avian *Paramyxovirus type 1* (APMV-1) virus [14]. NDV causes a deadly infection in over 250 species of birds, both domestic and wild, resulting in substantial losses to the poultry industry worldwide.

In fact, NDV naturally infects via respiratory and alimentary tract mucosal surfaces. In laying flocks, a sudden drop in egg production with a high proportion of eggs laid with irregular (soft) or misshapen shells are often early signs of the disease. Severe virus infection may lead to sudden death. After lesions, edema of the interstitial or peritracheal tissues of the neck may be presented, especially near the thoracic inlet. The symptoms are variable, depending on the virus strain, bird species, concurrent disease, and preexisting immunity [13, 14].

Newcastle disease virus (NDV) strains can be divided into three different pathotypes based on their virulence and severity of disease. Highly contagious velogenic strains are divided into viscerotropic and neurotropic velogenic strains. Viscerotropic velogenic viruses are responsible for acute lethal infections, resulted haemorrhagic, and necrotic lesions in the intestines of dead birds. Whereas, neurotropic velogenic viruses cause high mortality, follows with respiratory and neurological disease, but absence of gut lesions. Mesogenic strains resulted in respiratory and nervous symptoms causing moderate mortality; while lentogenic NDV strains cause mild infections of the respiratory tract in adult birds and are considered of low virulence [14].

Infection of NDV in the host cells is depending on two glycoproteins embedded in the viral lipid membrane, which are hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins that assist in virus infection. Initially, NDV infection ensues by attachment to the target cell through HN protein and sialic acid-containing receptors [15]. Upon adsorption of the HN to its cellular receptors, NDV undergoes conformational changes, which in turn triggers F protein conformational change and releases the fusion peptides to fuse the viral and cellular membranes. The F glycoprotein precursor (F_0) was proteolytically cleaved to the disulfide link and formed infectious particles, F_1 and F_2 [15, 16]. Finally, the penetration of NDV to target cells is by the endocytosis process [17].

5. Potential of Newcastle Disease Virus in Cancer Treatment

Like other viruses, NDV infects the host cells and then replicates itself. Scientists are interested in NDV because it can replicate itself more quickly in human tumor cells than in normal cells and cause oncolytic effects [18]. The NDV strains can replicate up to 10000 times better in human neoplastically transformed cells than in most normal human cells [19, 20]. The selective effect was probably due to the host restriction of V protein and virus-induced cytokines (IFN-γ and TNF-α) [21, 22]. The majority of tumor cells could be infected by NDV, and the viral replication within was detected by the increase of viral antigens on the cell

surface [23]. Thus, theoncolytic effect of NDV on human tumor cells is validated.

The first report of the application of NDV to treat human cancers was in the early 1950s, when adenovirus and NDV were injected directly into uterine carcinoma, resulting in partial necrosis and sloughing, but followed by regrowth [24]. This might be due to the production of neutralizing antibodies that inhibited the oncolytic activity of NDV. After that, many reports showed the possibility of NDV as a therapeutic agent in cancer treatment, from studies both in mouse models and in human clinical trials which showed favorable results [22, 25, 26].

The advantages of using NDV in cancer treatment are summarized as below.

(1) Ability of virus to bind to the tumor cell surface via its HN glycoprotein.

(2) Virus replicating in infected tumor cells leads to an enhanced expression of viral antigen on tumor cell surfaces.

(3) Ability of virus to induce synthesis of cytokines, like IFN and TNF, as well as stimulates production of heat shock proteins, adrenocorticotropic hormone, and tissue inhibitor of metalloproteases.

(4) Pleiotropic immunostimulatory effects of virus as the virus can augment the effects of T helper (T_H) cells, cytotoxic T lymphocyte (CTL), NK cells, and macrophages.

(5) Oncolytic activity or direct killing of tumor cells in treated patients.

(6) Rapid growth of virus in tumor cells.

(7) The virus is not pathogenic to humans.

6. Immunostimulatory Property of NDV

Although NDV causes direct oncolysis effects on tumor cells, NDV has the ability to modulate the human immune system (Table 1). Zorn et al. [27] showed that cellular cytotoxicity of peripheral blood mononuclear cells (PBMC) was enhanced significantly, after coincubation of NDV with effector cells. Throughout the study, NK cells were found to be the predominant mediator of lysis.

Indeed, NDV had been found to stimulate the host immunity to produce cytokines, such as IFN-α,IFN-β, TNF-α, and IL-1, which in turn leads to the activation of NK cells, macrophages, and sensitized T cells [19, 28, 29]. According to Fournier et al. [21], paracrine stimulation of IFN responses is through either exposure of viral HN proteins or by viral RNA. This virus-induced IFN-α/β is a potent inducer of NK cell-mediated cytotoxicity through induction of TNF-related apoptosis-inducing ligand (TRAIL) [30, 31]. Therefore, activated NK cells are considered as important contributors to innate defense against viral infections. Besides, IFN-α/β is also important in the generation of CTL activity [32]. The IFN-α released also functions for stimulation of cell-mediated cytotoxicity [33].

Human NK cells can be activated by NDV and lead to augmentation of antitumor cytotoxic activity towards tumor cells. Activated NK cells exert significant *in vitro* bystander antitumor activity, when stimulation cultures are performed on human tumor cell monolayers [34]. Adaptive transfer of the stimulated culture into immunodeficient mice bearing human breast carcinoma has resulted in tumor regression [34]. According to Jarahian et al. [35], NK cells can be activated through direct interaction between the HN viral glycoprotein and sialic acid residue containing in the cell surface. In fact, HN has been found to be a potent inducer of IFN production by human PBMC and is capable of upregulating the TNF-related apoptosis inducing ligands (TRAIL) [36]. Thus, activated NK cells are capable of stimulating cytokines secretion, such as IL-2, IFN-γ, and TNF-α, further influencing and activating other immune cells' functions. Importantly, NK cells display its ability to kill tumor cells independent with MHC class I molecule expressed on target cell surface. Therefore, it is acceptable to speculate that activated NK cells have more cytolysis effect against tumor cells.

NDV infection results in potent upregulation of major histocompatibility complex (MHC) class I molecules, antigen recognition molecules (HLA), and cell adhesion molecules (intracellular adhesion molecules (ICAM)-I and lymphocyte function-associated antigen (LFA)-3) on the tumor cell surface [37, 38]. Moreover, NDV infection leads to an increasing T cell costimulatory activity; consequently, enhanced cytotoxic potential of effector cells [37, 39, 40]. NDV-infected melanoma cells not only completely restored the proliferative response of the T helper (T_H) cells, but also prevented induction of anergy [40]. In addition, NDV induces production of various cytokines (IFN-α) as well as chemokines (RANTES and IFN-γ inducible protein 10 (IP-10)), finally undergo apoptosis [33, 39]. These chemokines function to chemotaxis as well as influence the activation status and cytotoxic activity of various immune cells [36].

Recently, it was shown that double-stranded RNA (dsRNA) is recognized by dendritic cells that have high expression of toll-like receptor (TLR)-3 that lead to maturation, activation, and protection [41–43]. NDV can activate macrophages and upregulate various macrophage enzymes, such as adenosine deaminase (ADA), inducible nitric oxide synthase (iNOS), lysozyme, and acid phosphatase. Through danger signals, activated dendritic cells promote cross-priming of T cells [33, 44, 45]. Activated dendritic cells increase their expressions of costimulatory molecules and stimulate T-cell response [43]. Also, NDV induces production of antitumor effector molecules, like nitric oxide (NO) and TNF. NDV administration also induce interferon (IFN) secretion,which further enhanced the phagocytosis of opsonized erythrocytes by mouse peritoneal macrophages [46]. The enhancement of phagocytosis activity might correlate with the stimulation of NO synthesis and the activation of NF-κB in macrophages which have important roles in mediating cytotoxicity [29]. Some encouraging results obtained by using NDV-activated macrophages to treat mammary carcinoma and lung carcinoma *in vitro* [47].

Table 1: Summary of the immunological properties of NDV.

Immunity	Consequences
Innate immunity	Apoptotic bodies lead to dendritic cell activation: augmentation of macrophage phagocytosis ability.
	Chemokines induction (RANTES and IP-10): stimulates chemotaxis, as well as recruitment of monocytes and T cells.
	Virus progeny resulted in monocyte activation: increased synthesis of NF-κB, NO, TRAIL, and augmentation of cytotoxic effect.
Adaptive immunity	T cells costimulation: upregulated MHC molecules expression, enhanced antigen presentation, and increased expression of cell adhesion molecules, such as ICAM-1 and LFA-3 molecules.
	Expression of viral HN molecules: increased production of IFN-α and TRAIL.
	Presence of double-stranded RNA: stimulation of TLR-3, IFN-α and heat shock protein expression.

Also, NDV exerts an immunostimulatory effect on monocytes. Upregulation of TRAIL mediated the tumoricidal activity of human monocytes, upon stimulation by NDV [33]. After 14 hours of coincubation, activated monocytes exerted antitumor cytotoxic activity towards TRAIL-sensitive tumor cells. Meanwhile, virus-stimulated PBMC mediated antibody-dependent cellular cytotoxicity (ADCC) through the Fc receptors of the antibody expressed.

In summary, NDV has very strong immunostimulatory properties for the generation of antitumor immune response. Through direct contact with effector cells, NDV caused cell activation, proliferation, and development. Besides, the cytokines produced also play an important role in the augmentation of immune responses.

7. Clinical Experience with Different Strains of NDV

The NDV strains that have been most widely evaluated for the treatment of human neoplasms are the nonlytic strain Ulster, as well as, the lytic strains MTH68/H, PV-701 and 73-T. Different virus strains may show various degrees of cytotoxic effects and viral production. When a nonlytic NDV strain is used to infect monolayer tumor cells, production of noninfectious viral particles was observed; in contrast, lytic NDV strains caused production of infectious particles that can infect other tumor cells, thus leading to an amplification of the viral load. Besides, infection of lytic NDV strains resulted in syncytium promotion and plague generation on tumor cell monolayers. The nonlytic strain Ulster showed stronger cytotoxic effects against colon carcinoma; in contrast, the lytic strain Italien caused effective killing of human melanomas [48].

Moreover, NDV possessed cytotoxic effects on tumor cells through two important components: exposure of the viral HN protein to the antigen-presented or tumor cell surface, facilitating the interaction between immune cells and tumor cells [49] and local induction of cytokines (type I IFN), which function for cell migration, activation and differentiation [32]. This statement was proven by the study by Li et al. [50], where the recombinant fowlpox virus which expressed NDV viral HN gene had enhanced cytotoxic effect on B16 tumor cells. *In vivo* vaccination caused the percentage of CD4 and CD8 T cells markedly increased, and also enhanced tumor-specific CTL activity. In addition, higher level of IFN-γ was secreted by T cells from the immunized mice that indicated the recombinant virus promoted T_H1-dominant response [50].

There are different conceptual applications of NDV in cancer and disease treatments like

(1) use for tumor selective cytolysis (oncolysis) [19];

(2) use of NDV as an adjuvant in a tumor vaccine for stimulation of CTL and delayed-type hypersensitivity (DTH) responses after antitumor vaccination [20];

(3) use of NDV for nonspecific immune stimulation and induction of cytokines, like interferons [36, 39];

(4) use of NDV as viral vector for delivering therapeutic genes [51];

(5) use of NDV as vaccine vector for immunization against emerging pathogens [52].

8. NDV Oncolysate with Strain 73-T

Viral oncolysate was prepared by using primary explants of human tumor cells incubated with NDV [53]. Since the mid-1950s, NDV lysate started to be administered to cancer patients. The oncolysate functioned to augment antitumor immunologic responses towards metastatic disease. Administration of oncolysate to the patients resulted in increased of T lymphocyte percentage and enhanced cytotoxicity.

Initially, viral oncolysate was prepared by using the lytic NDV strain 73-T [24]. The virus was obtained by passaging of NDV strain 379-SI on Ehrlich ascites tumor cells *in vitro* for 73 times and *in vivo* for 13 times, in a reason to eliminate the neurotrophic properties of the strain. NDV strain 73-T has the ability to replicate in human tumor cells, causing cell-cell fusion, syncytium formation and tumor cell death [54]. *In vitro*, the virus kills many human cancer cells, such as fibrosarcoma, osteosarcoma, neuroblastoma, cervical carcinoma, Wilm's tumor, and so on [55]. In addition, the oncolytic potency of NDV strain 73-T was demonstrated in mice with human tumor xenograft models. Intratumoral and intraperitoneal injection of NDV strain 73-T caused durable, complete tumor regression in athymic mice bearing human neuroblastomas and fibrosarcoma xenografts [55]. More than 67% inhibition of tumor growth was observed, upon virus infection. Another study by Phuangsab et al. [56]

showed locally administered virus was able to inhibit tumor growth (77 to 96%) in several carcinoma xenografts in mice, including cancer of epidermoid, colon, lung, breast, and prostate xenografts. Furthermore, complete tumor regression was observed in 9 of 12 mice bearing IMR-32 neuroblastoma tumor xenografts, after a single intraperitoneal injection of NDV. Most important of all, this strain did not cause any adverse effect on normal human cells [55].

The first clinical documentation of NDV activity was reported by Cassel and Garrett [24], involving one cervical cancer patient. In this paper, the 2.4×10^{12} virus particles were injected directly into the tumor demonstrated intratumoral regression of the local cancer and also a distant malignant lymph node [24]. Partial necrosis and sloughing were observed, but this was followed by tumor regrowth. Following this study, a phase II clinical trial with administration of viral oncolysates was performed for patients with malignant melanoma [57]. As a result, 6 out of 13 patients showed a decrease in the size of the skin nodules and/or lymph node lesions.

Cassel et al. [58] did phase II clinical trials comprising of 32 patients at high-risk stage II melanoma, viral oncolysate was administered, following surgical excision of metastatic nodes. After the treatment, it was observed that progressive disease occurred in only 6%, 8%, and 12% of patients. In another clinical trial which involved 83 patients at stage II malignant melanoma, NDV oncolysate was applied as an immunotherapeutic agent in postsurgical management [59]. The patients were observed for at least 10 years with over 60% are survived and free of recurrent disease. The survival rate was significantly higher than historical controls. This indicated that the NDV oncolysate was helpful as an adjuvant to surgery in the management of malignant melanoma.

Furthermore, a 15-year follow-up phase II clinical trial initiated in 1975 on patients with stage III malignant melanoma treated with NDV oncolysate indicated more than 60% of ten-year survival without any adverse effects. Continued analysis of the trial showed 55% of overall fifteen-year survival. Extended survival was observed among patients who displayed an increase in the number of $CD8^+$ $CD56^+$ T lymphocytes, as these cells provide effective immune defense against tumor cells. In addition, the increased cells also produced large amounts of cytokines, like TNF-α and IFN-γ, to aid in cytotoxicity [60].

9. Autologous Tumor-Cell Vaccine (ATV) with NDV Strain Ulster

Besides NDV oncolysate, a new strategy for the design of a human tumor vaccine was developed by Liebrich et al. [61]. The tumor vaccine consisted of patient-derived autologous live tumor cells inactivated by irradiation and then infected by the nonlytic NDV strain Ulster. Then, the vaccine was stored in liquid nitrogen until application. The idea of autologous tumor-cell (ATV) vaccine had come from the study of virus-modified Esb cells to treat lymphoma in animal models [62, 63]. Viral modification leads to an increase of tumor cell immunogenicity [62]. The vaccine was used as challenge for a new antimetastatic therapy strategy, as chemotherapy drugs became less effective. Postoperative vaccination with virus-modified ESb cells was able to give protection from metastases in more than 50% of syngeneic mice [63]. The surviving mice developed long-lasting protective immunity towards lymphoma, due to the immune T-cell memory system. Schild et al. [64] had described the enhancing of T-cell immune activity, upon immunization using NDV-modified tumor cells. Also, the production of cytokines, such as IL-2 and IFN-α/β, was increased after antigen stimulation. These cytokines were essential for the generation of tumor-specific CTL activity [32].

To prepare the ATV-NDV vaccine, nonlytic strain Ulster is used in the culture of patient-derived tumor cells. The selection of this strain was based on several reasons. First, it is an RNA virus, which cannot integrate into the host cells' genome. NDV replicates selectively in the tumor cells, but not normal cells [24, 54]. Besides, NDV possesses pleiotropic immunomodulatory properties [27, 33]. There are a lot of successful test cases in preclinical and clinical studies, without any severe side effects [55, 58, 59, 63].

NDV strain Ulster has a monocyclic abortive replication cycle in tumor cells [20]. The virus first is adsorbed on to the tumor cells, taking about an hour for binding. The virus is allowed to remain in the body for a generation for effective immune responses, most probably T-cell-mediated immunity. Direct contact of virus with immune cells will affect cell proliferation and activation status. As viral replication takes about 10 to 50 hours in tumor cells, it is sufficient for the generation of DTH skin responses [20].

Clinically, the ATV-NDV was tested in 23 patients with colorectal liver metastases. Vaccination was applied to the patients after they underwent liver resection [61]. As a result, the patients showed increased recurrence-free intervals and DTH skin reactivity. In another study, favorable results were obtained by using ATV-NDV, comprising of a dose of 1×10^7 human colorectal tumor cells together with 32 hemagglutination units (HAU) of NDV, intracutaneously administered to colorectal cancer patients [65]. After four vaccinations at two-week intervals, the DTH responses were increased at distant sites. This indicated an augmentation of tumor reactive T lymphocytes.

The study was continued by a clinical study in 20 colorectal cancer patients after surgical resection of the tumor [66]. The ATV-NDV vaccine was prepared with different numbers of tumor cells ranging from 2×10^6 up to 2×10^7 cells, and NDV concentrations from 4 to 64 HAU. Overall, 16 patients responded with a DTH skin response after vaccination. After 24 hours, optimal skin reactions were observed with 1×10^7 tumor cells infected with 32 HAU of NDV strain Ulster [66, 67]. This means that the presence of low amounts of antigen was enough to induce local memory immune response of cancer patients. Then, phase II clinical trial was undertaken in postoperative active-specific immunization (ASI) with ATV-NDV to 23 colorectal carcinoma patients following resection of liver metastases [67]. Encouraging results were obtained as the vaccinated group experienced lower recurrence rate, compared to a historically matched control group.

Long-term survival rate of vaccinated patients was reported by Kirchner et al. [68]. ATV-NDV was used as a surgical adjuvant vaccine for 208 patients with locally advanced renal cell cancer. Vaccination caused a median disease-free survival of 21 months, higher than the historical controls [68]. With a two-year followup, the analysis showed that only 10 relapses (18%) among the patients, along with a median followup of 39 months. Another study involved a group of 48 cancer patients; the vaccinated patients experienced 97.9% of two-year survival rate, higher than the historical control [69]. Besides, encouraging results were obtained in a nonrandomized study involving 23 glioblastoma patients vaccinated by ATV-NDV [70]. The results showed that vaccination lead to improvement of the median survival rate, significantly higher than the control group.

Another study in China indicated the efficiency of NDV vaccination as adjuvant after tumor cell resection. A total of 310 colorectal cancer patients with resection received ATV and NDV strain LaSota vaccine as adjuvant [71]. The results showed that advanced tumors of the digestive tract significantly regressed upon vaccination. The one year survival rate of the vaccinated patients was 96%. Followup of the vaccinated patients showed 56.5% in seven-year survival rate, compared to the control group (43.42%). After vaccination, the total effective rate (complete and partial remission) was 24%, including one case of complete tumor remission [71]. Most important of all, the vaccine augmented immune activities by increase the number of NK cells.

In a colorectal carcinoma study, the high quality of the ATV-NDV vaccine caused a 25% increase in the 5-year survival rate [72]. Similar results were obtained in a recent study involving 51 colorectal cancer patients with liver metastasis [73]. A total of 6 doses of vaccination showed improvement of metastasis-free survival rate. Vaccinated patients had better survival rate (48% above the control group). All vaccinations were tolerated [73]. Only 16% of the vaccinated patients experienced minor side effects, including local erythema and itching at the injection site. A single case reported headaches on the first vaccination day, but it did not recur for the subsequent vaccinations.

The quality of the ATV-NDV vaccine is critical for antitumor efficiency. This was proven in the study by Schirrmacher [72] that the high quality vaccine showed 36% higher efficacy than the low quality one, in terms of five-year survival rate in an advanced breast cancer study. Hence, improvement of the quality and efficacy of ATV-NDV vaccine was carried out. Ockert et al. [69] modified the vaccine preparation step by enrichment of the tumor cells through Percoll centrifugation, followed by the removal of tumor-infiltrating leukocytes (TIL) using immunomagnetic beads. Besides, improvement of antitumor efficiency of ATV-NDV vaccine could be achieved in another way, by the addition of recombinant IL-2 [74, 75]. Vaccinated patients benefited with improved survival rate, with three-year and five-year survival rates of 67% and 61%, respectively [75]. A significant number of patients had increased tumor-specific T lymphocytes, even after 5 to 6 years after vaccination, thus conferring antitumor immunity. In another study, the antitumor efficiency of ATV-NDV was enhanced with the aid of recombinant bispecific hybrid antibodies [37]. The antibodies-coated ATV-NDV caused upregulation of T cell activation markers (CD3 and CD28) within 24 hours.

In summary, the ATV-NDV vaccine appeared to be feasible and safe to treat advanced cancers such as colorectal cancer, breast cancer, ovarian cancer, glioblastoma, kidney cancer, and head and neck cancer [65, 70, 72, 75, 76]. Continuous efforts for the improvement of the tumor vaccine quality are carried out in order to improve the prognosis for survival of vaccinated patients.

10. PV701 Strain

PV701 strain is a nonrecombinant, replication-competent NDV isolated by investigators at Pro-Virus Inc. (Gaithersburg, USA). It is a naturally attenuated, triple-plaque-purified isolate from the mesogenic NDV strain MK107. The broad-spectrum oncolytic activity of this virus strain is probably due to tumor-specific defects in the IFN antiviral response. This NDV strain is considered to be tumor selective, as it is sensitive to most human cancer cell lines, depicting a two-to-four log order higher sensitivity than to normal cells [19].

The oncolytic effect of NDV strain PV701 was reported in the study by Lorence et al. [77]. Intravenous administration of PV701 in a dose-escalation study in tumor-bearing mice produced partial tumor regressions at doses as low as 6×10^5 plague forming unit (pfu). More than 80% of the mice developed complete tumor regressions at doses up to 6×10^8 pfu. The antitumor response was associated with evidence of viral replication.

These encouraging results led to initiation of a phase I clinical trial to intravenously administer PV701 strain to advanced solid cancer patients. Intravenous administration of NDV strain PV701 vaccine was done on 79 patients with solid tumors [19]. A maximum tolerated dose (MTD) following a lowed initial desensitized dose at 12×10^9 pfu/m^2; and subsequent infusions were increased 10 folds, tolerated up to 120×10^9 pfu/m^2 [19, 78]. Further dose escalation on the patients would lead to hypotension. In this study, the virus strain caused regression of advanced solid cancers, without observed cumulative toxicity [19]. One patient's squamous cell cancer on his tonsil was completely eliminated after vaccination. Measurable tumor reductions were seen in another seven patients with diverse malignancies [19]. Unfortunately, one possibly treatment-related death involved a renal cancer patient with lung metastatic [19]. Postmortem revealed inflammation occurred in lungs, suggesting rapid tumor lysis leading to compromised pulmonary function after vaccination.

More work is required to improve patient's tolerance. Therefore, following the phase I trial, some modifications were performed by Laurie et al. [79]. Two-step desensitization was implemented by using two dose increments, before high repeat dosage. As a result, a patient with anal carcinoma experienced tumor regression and four patients had stabilization of their disease for more than 6 months. Primarily, the first dose of desensitization allowed higher tolerance of

subsequent doses [78, 80]. Hotte et al. [78] modified the vaccination scheme by introducing slow infusion, in order to improve patient tolerance. The MTD for initial slow infusion was 24×10^9 pfu/m^2 and subsequent infusions were safely escalated to 120×10^9 pfu/m^2 [78]. A total of 54% patients survived over 4 months of progression-free intervals, after vaccination. 15 out Of 18 treated patients, developed stable disease, including 4 major and 2 minor tumor responses [78]. Phase II continuous studies are ongoing for patients with cancer resistant to conventional modalities.

In summary, NDV strain PV-701 well tolerated an intravenous dosage of at least 3×10^9 infectious units and at least 4×10^{12} infectious units by intratumoral route [78]. The developments of two complementary strategies, namely, desensitization and slow infusion, have led to improvement of the vaccine with reduced toxicity. So far, favorable results were observed when using the virus to treat diverse human cancers. Generally, the mild side effects observed were flu-like symptoms, tumor-site-specific adverse events, and infusion reactions [19, 79, 80]. The adverse effects were dose-dependent. Of the seven patients with noncardiac chest and/or back pain, five among them received highest dosage [79]. Other typical side effects observed in some patients were leucopenia and neutropenia. Occasionally, virus infection was associated with transient thrombocytopenia and diffuses vascular leakage [19]. Presence of viral particles was observed in the tumor tissue of vaccinated patients, but not in heart, lung, kidney, liver, or brain tissue [19, 77]. Virtually, most of the vaccinated patients developed neutralizing antibodies towards NDV strain PV701. Besides, viable virus was recovered from the urine of vaccinated patients, and rarely in sputum, but the virus recovery did not persist and was cleared within 3 weeks.

11. MTH-68/H Derived from the Hertfordshire Strain

The mesogenic NDV strain Hertfordshire was isolated in England in 1933, and later known as Herts'33. Early study by Alexander et al. [81] reported the cytopathogenicity and production of NDV stain Herts'33 progeny in animal cell lines. Virus infection at low multiplicities caused cell fusion within 24 hours; while at high multiplicities, the effects were induced within 3 hours after infection. Among these cells, virus replication happened in MDBK cells, chicken embryo cells, and baby hamster kidney clone (BHK-21) cells. Meanwhile, NDV caused more cell lysis to Madin-Darby bovine kidney (MDBK) cells, as lactate dehydrogenase (LDH) was released in large amount after 24 hours of infection.

The first intensive use of NDV strain Hertfordshire for cancer treatment was pioneered by Csatary [82]. He developed a novel virus strain named MTH-68, which means "More Than Hope 1968." Since then, many researchers started to investigate its anticancer ability. MTH-68/H strain has the ability to cause significant regressions of human tumor cell lines in varying degrees, such as PC12, MCF-7, HCT116, DU-145, HT-29, A431, HELA, and PC3 cells. Activation of caspase 8- and 9-induced apoptosis on the virus-infected cells, irrespective of their p53 conditions [83]. Indeed, MTH-68/H was the most potent IFN-α inducer among all NDV strains tested [84]. Besides, this NDV strain also has the ability to induce nitric oxide (NO) and to increase the macrophage population in treated rats, resulting in enhancement of antitumor effects [85].

Clinically, in one placebo-controlled trial, the MTH-68 vaccine was administrated to 33 patients with advanced cancers in the way of inhalation twice weekly [25]. Favorable responses occurred in a total of 18 patients (55 %) compared to 2 patients in the placebo group (only 8 %), as the tumor stably regressed [25]. Seven vaccinated patients survived more than 2 years, whereas none from the control group.

An individual case of vaccination of NDV strain MTH-68/H to a 14-year-old patient with high grade glioblastoma was reported in 1999. The patient received adjuvant chemotherapy, after tumor resection and radiation therapy. Inefficient tumor clearance forced the patient to receive NDV vaccine. During that time, the patient continued receiving tamoxifen as adjuvant. As a result, the tumor progressive shrunk by about 95% from the scan, without any neurotoxic effects [86]. This breakthrough case of complete remission of tumor indicates that NDV vaccine may be a potent cancer treatment.

A recent study showed that ultraviolet light (UV) inactivated MTH-68/H was a potent interferon-α inducer and could induce human PBMC antitumor activity *in vitro* [84]. Therefore, Apostolidis et al. [84] utilized locoregional therapy for the treatment of liver metastases of luciferase-transfected murine CT26 colon carcinoma cells. As a result, NDV strain MTH68/H caused a significant delay in tumor growth and prolonged survival, without severe side effects. Loss of body weight did not occur among the vaccinated mice [84].

12. Other NDV Strains

The NDV strain HUJ (OV001) is a lentogenic strain, which is highly purified, isolate originally derived from naturally attenuated B1 NDV vaccine strain. This strain has high selective cytopathogenicity to human and animal cancer cell lines. Virus infection leads to viral replication producing virus progeny. However, the virus progenies produced by the lentogenic strain are noninfectious, because of incomplete processing of the fusion (F) protein. Besides direct cytotoxic effects on target cells, NDV strain HUJ also induces cytokine-mediated events and augments the immune reactions [87].

In a recent phase I/II trial, NDV strain HUJ was administered intravenously to 11 patients with recurrent glioblastoma multiforme (GBM). Following biweekly maintenance therapy, one patient experienced stable disease after the first cycle of vaccination; and later, complete tumor remission with duration of 3 months. This might be due to the patient developed neutralizing antibodies in the early stage. Normally, neutralizing antibodies appeared within 5 to 29 days. Infectious NDV was recovered from blood, urine, and saliva samples and have a tumor biopsy sample. Administration of

strain HUJ caused mild side effects to the patients, including grade I/II constitutional fever and headache. Sometimes, the patients might experience neurological problems and thrombosis. Intravenous administration of NDV strain HUJ vaccine is well tolerated. The encouraging responses of stain HUJ warrant the evaluation of NDV in other cancers, besides GBM [87].

Another lentogenic strain, LaSota was also shown to induce antitumor cytotoxic effects of mouse macrophages by the production of TNF-α [33]. The anticancer activity of activated monocytes was attributed to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [33]. In the study by Liang et al. [71], vaccination of NDV strain LaSota was efficient to prolong colorectal cancer patients' life, with 96% patient survival after 1-year treatment with NDV immunotherapy. Moreover, the number of NK cells increased, and immune function obviously improved.

For NDV strain Italien, Ahlert and Schirrmacher [88] showed that this lytic strain could replicate in different human tumor cells, such as carcinoma of breast, squamous lung, kidney, melanoma, and lymphoma. Intratumoral injection of NDV strain Italien displayed high sensitivity to human metastatic melanoma xenotransplants in nude mice [48]. There was an MTD in mice in the range of 2000 HAU virus strain Italien [89]. However, nonlytic strain Ulster showed stronger cytotoxicity effect on a CT26 colon carcinoma model [48]. This suggested that the antitumor effect on different tumor cells was correlated with the NDV virulence. Importantly, NDV virus replication did not happen in normal cells, including resting T lymphocytes and normal chicken liver cells [20].

13. Genetically Modified NDV Strains

Recently, the antitumor efficacy is improved with the new emerging idea of using recombinant NDV with a therapeutic gene. To enhance the immunostimulatory properties of NDV, the *IL-2* gene was introduced into the viral coding sequence [90, 91]. Thus, virus infection leads to production of IL-2 and initiates immunological effects, including T-cells activation and IFN-γ production. *In vivo*, colon carcinoma tumor-bearing mice treated with recombinant NDV-IL-2 showed significant tumor regression and T-cells infiltration 90. *Ex vivo*, the NDV-IL-2 oncolysate resulted in activation of tumor-specific CTL and memory T cells [90].

In another study, Janke et al. [51] inserted a recombinant granule-macrophage colony-stimulating factor (GM-CSF) as an additional transcription unit into NDV, in order to augment antitumor immunity. Vaccination of recombinant virus stimulated human PBMC to exert antitumor effects. Furthermore, higher synthesis of IFN-α was observed, most probably contributed by activated monocytes and dendritic cells [51]. Indirectly, T_H immunity was enhanced also.

Later, generation of a recombinant NDV expressing influenza NS1 protein, a protein exhibiting IFN-antagonist, was reported. As a result, the virus enhanced its ability to form syncytia and lysis effect on tumor cells in human and animal models, thus resulted in higher overall long-term survival. Besides, vaccination of recombinant NDV led to high degree of T-cell infiltration, suggesting the generation of the tumor-specific CTL response [92].

Another recombinant NDV strain was designed by Bian et al. [89], in which the virus was modified by preincubation with a recombinant bispecific protein (IL-2 receptor). A new binding site was introduced to the virus; which enhanced its interaction to tumor-associated target. Higher virus replication efficiency was noticed in the Eb-M7 (IL-2 receptor positive) syngeneic tumor-bearing mice [89]. Administration of modified NDV revealed that side effects were reduced without affecting the antitumor activity.

In conclusion, genetically modified NDV strain may have not only antitumor effect, but also augmented immunomodulatory effect. Previous study showed that the recombinant NDV had high efficiency to deliver therapeutic effects, without affecting oncolytic activities. This proved that NDV is a high potent vector. Importantly, the virus does not cause pathogenicity.

14. Involvement of Malaysian Isolates of NDV Strains in Cancer Research

The oncolytic effects of several local NDV strains, including AF2240, 01/C, Ijuk, S, F, V4-UPM strains, on human cancer cell lines, such as CEM-SS (T-lymphoblastic leukemia cells), HT-29 (colorectal cancer), MCF-7 and MDA-231 (breast cancer), and HL-60 (acute promyelocytic leukemia) had been reviewed by Omar et al. [26]. Othman et al. [93] reported that NDV AF2240 selectively targeted estrogen dependent cancer cells, such as MCF-7 breast cancer cells. F strain displayed significant oncolytic effects on MDA-231 and MCF-7 cells, but Ijuk killed MDA-231 cells only. A study by Zulkifli et al. [94] showed that the V4-UPM strain displayed oncolytic effects against human malignant gliomas (DBTRG.05MG and U-87MG) in tissue culture. Complete regression of U-87 MG gliomas tumor-bearing mice was observed also. *In vivo*, intratumoral treatment using NDV strain AF2240 in human breast cancer cell xenotransplanted mice caused partial regression [95]. The virus was detected in the breast tumor sites [96]. However, the virus was disseminated to normal organs (e.g., liver), following intratumoral infusion. Virus dissemination may affect the gene therapy efficiency by reducing transgene expression in the tumor, by accumulating in the normal tissues [97].

15. Safety of NDV Administration as Anticancer Agent

Previous studies of utilizing NDV strains as anticancer agent have resulted in encouraging results. Scientists are interested in the therapeutic effect of NDV, because of its tumor-selectivity [18]. NDV strains can selectively replicate up to 10000 times better in tumor cells, but not in normal cells [54]. Numerous reports had shown that the virus cannot replicate in nontransformed cells, such as fibroblast cells, resting T lymphocytes, and normal primary culture

[20, 54, 55, 83]. Besides, NDV is an immunostimulatory agent, as it can induce antitumor activities of a variety of effector cells, including NK cells, macrophages, and CTL [32, 33, 36, 47].

Prior to human clinical trials, the public may question the safety issues of the vaccine. There is an extensive safety database for NDV, primarily from dose escalation trials. All vaccinations are well tolerated in human studies. According to Pecora et al. [19], oncolytic NDV strain is well tolerated in doses of at least 3×10^9 infectious units by the intravenous route and at least 4×10^{12} infectious units by the intratumoral route. While, the MTD of initial desensitized dose at 12×10^9 pfu/m^2; subsequent infusions were tolerated up to 10 folds, at 120×10^9 pfu/m^2 [19, 78]. Up to now, there is no report on accumulative toxicity associated with repeating vaccinations with NDV as evidenced by one cancer patient who received over 30 courses of PV701 without recording any adverse events. Basically, the virus was able to clear from the body within three weeks [19].

Also, the safety and efficiency of NDV vaccination is deduced from improvement of the cancer patients' survival rate. Ockert et al. [69] had reported the five-year survival benefits in phase II trials involving patients with locally advanced colorectal carcinoma. Another study by Karcher et al. [75] revealed 61% of vaccinated patients with stage III and stage IV head and neck squamous cell carcinoma experienced increase of five-year survival rates. So far, many cases of tumor regression had occurred in NDV-vaccinated patients. In a glioblastoma patient with resection, complete remission was observed after several months' vaccination with ATV-NDV [70]. The therapy therefore has promising antitumoral activities in patients.

In addition, NDV vaccination augmented human anti-tumor immunity, especially tumor-specific CTL activities, increased DTH responses [61, 65, 67]. Literally, the best DTH skin reaction was obtained using a vaccine, comprising of 10^7 tumor cells and 32 HAU NDV [66, 67]. It caused a median induration of 8 mm on the vaccination site. The DTH responses to the vaccine increased throughout repeated vaccinations. Especially encouraging is that the vaccinated patients acquired neutralizing antibodies to NDV [19].

In fact, NDV may infect human and cause mild side effects. Through the experience with farmers and laboratory researchers, NDV infection produces only minimal disease. The general side effects displayed on vaccinated patients are conjunctivitis, laryngitis, hypotension, and mild flu-like symptoms, including fever (up to 38°C), chills, tiredness, headache, muscle pain, and weakness [69, 72, 73]. On the vaccination sites, erythema, swelling, induration, and itching were observed [69, 73]. Other typical side effects observed in some patients were leucopenia and neuropenia. Occasionally, virus infection was associated with transient thrombocytopenia and diffuse vascular leakage [19]. These side effects are temporary and disappear in 1 to 2 days after vaccinations.

Unfortunately, one possibly treatment-related death involved an old patient with renal carcinoma metastatic to the lungs and compromised pulmonary function [19]. Postmortem revealed inflammation occurred in the tumor-bearing lung, suggesting rapid tumor lysis leading to fatal respiratory failure after desensitized vaccination. This raises the safety challenge of NDV vaccine administration in cancer patients. Hence, the US Food and Drug Administration (FDA) has not approve NDV as a cancer treatment until today.

In conclusion, the safety of NDV strains as anticancer agent has been consistently high with low toxicity. Although NDV therapy causes mild side effects, the responses are negligible as the quality of life of the vaccinated patients is not affected in negative manner. Despite applications in thousands of people, NDV vaccination has not caused any severe adverse effects. This explains the renewed interest in NDV as an anticancer agent [18].

16. Conclusion

Based on all the previous research, NDV is safe and feasible to be used as a therapeutic agent. More systemic investigations are necessary to enhance the quality and efficacy of NDV vaccine. Further testing or even preparation of a DNA vaccine may be required to confirm the safety of virus administration and to improve the public's acceptance. In short, NDV can be a potential alternative adjuvant in cancer treatment.

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