Role of nontoxigenic *Clostridium novyi* in solid tumor therapy

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Current treatments for most solid tumors include surgery, chemotherapy, radiotherapy, and so on. However, these methods have their own limitations; besides, it has been proved that solid tumors always possess relatively anaerobic areas that are closely related to tumor resistance and poor prognosis. Seeking new alternative treatments is urgent; trials using anaerobic bacteria to target this troublesome region were initiated years ago. Nontoxigenic *Clostridium novyi* (*C. novyi*-NT) possesses the capacity to colonize specific tumor causing tumor necrosis, and, most significantly, can be exploited to treat solid tumors combined with several current cancer therapies successfully. Undoubtedly, the application of *C. novyi*-NT in solid tumor therapy has been demonstrated as a novel and promising therapy.

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Introduction

Tumors are characterized by uncontrolled and invasive growth of cells [1]. Although the conventional therapies, including surgical resection, radiotherapy, and chemotherapy, are quite effective among cancer patients [2], there still exists a considerable number of patients, showing no response to those treatments because of the resistance to conventional therapies and tumor metastasis [3]. Because of limitations of the current therapies, a new wave of seeking alternative treatment has been accelerated. Many experimental methods of treating cancer have been developed, such as photodynamic therapy [4], gene therapy [5], telomerase therapy [6], noninvasive radiofrequency cancer treatment [7], and bacterial treatment [8]. Considering their efficiency, feasibility, availability, specificity, and toxicity, many methods still remain controversial. Nevertheless, among these methods, the advent of bacterial treatment has had a dramatic and significant impact on the development of cancer treatments [8–10]. The pioneer work of using bacteria as an antitumor agent can be traced to a century ago, when W.B. Coley, an American surgeon, was inspired by regression of tumor in cancer patients after an accidental erysipelas infection, and developed a vaccine, known as 'Coley toxins', to treat cancer patients and the vaccine successfully induced the regression of tumor [11]. The early success of Coley's trial provides the grounds for current research in bacterial therapy.

Experimental studies have demonstrated that certain species of the anaerobic bacteria, such as *Clostridium* and *Bifidobacterium*, showed the ability to reproduce selectively in tumor cells or within the tumor microenvironment and were able to induce tumor regression [9]. The principle of this utilization is based on the fact that solid tumors normally have hypoxic areas because of tumor overgrowth and immature, aberrant vasculatures [12]. In experiments, live nonpathogenic bacterial species such as *Bifidobacterium adolescentis* [13], which have been

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Table 1. Schematic overview of application of anaerobic bacteria in cancer therapy.

Genus	Species	Features	Therapeutic methods	References
Bifidobacterium	*B. longum*	Lactid acid-producing	Bacterial directed enzyme prodrug	[17–19]
	B. adolescentis	Difficult to store and handle		[20,21]
	B. infantis			[22,23]
Salmonella	*S. typhimurium*	Intracellular bacteria	Attenuated vaccine	
	S. choleraesuis	Facultative anaerobes Relatively high ratio of colonization in normal organs	Vector of exogenous gene encoding tumoricidal cytokines	
Clostridium	C. *sporogenes*	Strictly anaerobic bacteria	Immunoagents	[24–26]
	C. *oncolyticum*	Spore former	Vector of exogenous gene encoding tumoricidal cytokines	[27,28]
	C. *novyi*	Stable and easy to operate	Combined with conventional therapies	[29–31]
	C. *butyricum*	Available in intravenous injection	*Clostridium*-directed enzyme prodrug therapy	[32–34]
	C. *acetobutyricum*	Better oncolytic activity	*Clostridium*-directed antibody therapy	[35]

attenuated or genetically modified, were commonly utilized as tumoricidal agents to generate direct tumoricidal effect or provide tumoticidal molecules. In addition, bacteria can be exploited as delivery agents for cancer drugs and as vectors to carry tumoricidal agents or bacterial enzymes, such as VNP20009 was modified to express tumor necrosis factor-α and platelet factor 4 fragment [14]. The general overview of application of anaerobic bacteria in cancer therapy can be given as a framework below (Table 1). Compared with *Bifidobacterium, Clostridium* can be better preserved and systemically injected into experimental models in the form of spores, which showed much lower toxicity [15,16]. *Clostridium novyi* and its application in solid tumor therapy will be reviewed.

The selection of nontoxigenic *Clostridium novyi*

Dang *et al*. [29] screened 26 strains from *Bifidobacterium, Lactobacillus,* and *Clostridium* genera to identify their tumor colonization capacity. Among these strains, only two (*C. novyi* ATCC19402 and *Clostridium sordellii* ATCC9714) exhibited extensive spreading throughout the poorly vascularized portions of the solid tumors. When a large number of spores of *C. novyi* and *C. sordellii* were injected intravenously into B16 tumor-bearing mice, Dang *et al*. observed that *C. novyi* bacteria rapidly dispersed within tumor in 16 h and seemed to be restricted because they did not find any vegetative cells in the liver, spleen, kidney, lung, or brain of these mice. Although *C. novyi* showed particular affinity for the tumor and exerted a tumoricidal effect, all tumor-bearing mice died 16–18 h later. In order to eliminate the lethal toxicity caused by *C. novyi* infection, they successfully deleted the only gene encoding toxin located in phage by heating the *C. novyi* spores, and subsequently cloned the new strain, which was renamed *C. novyi*-NT (nontoxigenic *C. novyi*) for further investigation. The development of *C. novyi*-NT in solid tumor therapy can be generalized as follows (Fig. 1).

Following the modification, they injected the spores of *C. novyi*-NT intravenously into tumor-bearing mice; these spores retained the capacity of tumor colonization and significantly caused massive areas of tumor necrosis without any sign of bacterial germination in healthy organs. Nevertheless, the well-oxygenated rim of tumor was spared because of the sensitivity of *C. novyi*-NT to oxygen, which led to the recurrence of tumor [29].

A single intravenous injection of spores of *C. novyi*-NT exhibited such potent tumoricidal ability. Although it has not been fully understood so far, three possible explanations are possible. First, microbiologically, *C. novyi* is a motile, spore-forming, and obligate anaerobe that can cause gas gangrene [36,37]. As for *C. novyi*-NT, apart from its lethal toxicity, its other features were successfully retained [29]. Based on the current study of its genome [38], considerable information exists about the genes controlling sporulation, germination, and growth, as well as genes affecting spore coat architecture. Apart from this, several genes encoding proteins that may be quite important in tumor lysis were also identified. Second, we have seen the tumoricidal ability of *C. novyi*-NT spores when they were injected into nude mice bearing human xenografts. In experiments of *C. novyi*-NT spore treatment of immunocompetent mice bearing syngeneic murine CT26 tumors, 30% of them were cured and showed long-term survival. It has been proved that the antineoplastic effect was mediated by the immune response, induced by *C. novyi*-NT infection [26]. Moreover, cured animals rejected a subsequent challenge of the same tumor [25,26]. Similar results were observed in rabbits and dogs with certain syngeneic murine tumors [26,39]. Third, *C. novyi*-NT can also secrete extracellular proteins, such as phospholipase, and lipases, which may interact with host and thereby cause tumor lysis and induce antitumor immunity [38,40,41].

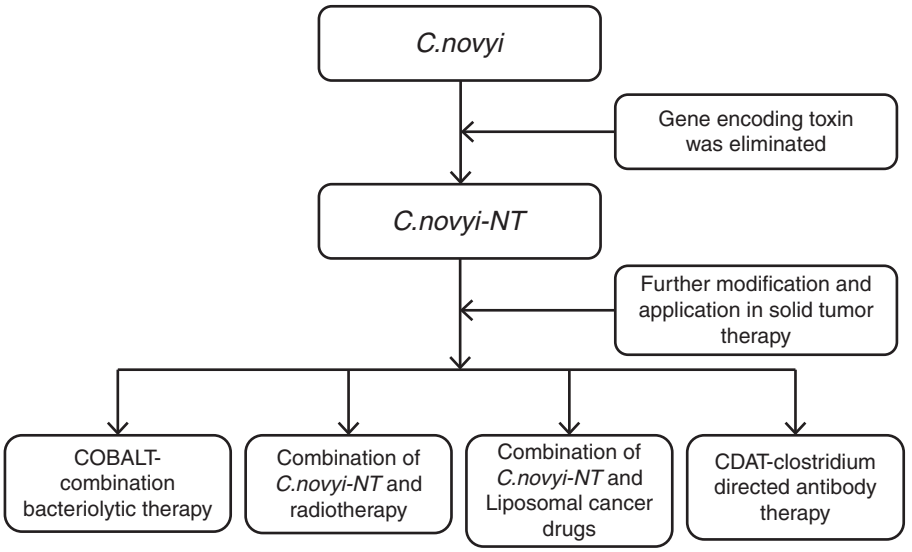

Fig. 1. Systemic overview of research progress of nontoxigenic *Clostridium* novyi (*C. novyi*-NT) in solid tumor therapy. CDAT, *Clostridium*-directed antibody therapy.

Interestingly, liposomase, a protein secreted by *C. novyi*-NT, has been shown to be very active when combined with liposomal cancer drugs to target solid tumors [33], and this will be discussed later.

Years of research have indicated that tumor resistance to conventional therapies (chemotherapy and radiotherapy) always results in poor prognosis and is closely related to the existence of low oxygen levels in solid tumors [42]. Moreover, apart from the obstacles of tumor angiogenesis [43], uncontrolled growth, and other characters, the new development of cancer therapies must overcome the limitation of specificity. Now that the spores of *C. novyi*-NT must germinate and propagate only under anaerobic conditions, the character of tumor hypoxia could be exploited [44,45] and thus, a new therapeutic strategy – combining *C. novyi*-NT with current cancer therapies – was proposed.

Combination of nontoxigenic *Clostridium novyi* spores with traditional cancer treatments

Following the strategy mentioned above, Dang *et al.* [29] first carried out the experiments of combining spores of *C. novyi*-NT treatment with conventional chemotherapeutic agents, which is called COBALT – combination bacteriolytic therapy. After the intravenous injection of *C. novyi*-NT spores to tumor-bearing mice, microtubule-binding agent D10, and DNA damaging agent MTX were given, respectively. The results confirmed the combination quite significant because much more extensive necrotic areas in tumor were observed both in gross inspection and histologically. In many mice, those necrotic masses eventually dissolved and disappeared, leaving the animals tumor free. Nevertheless, the high cure rate of COBALT was accompanied by negligible toxicity, which was considered to be correlated to tumor size (tumor lysis syndrome) [46]. Based on the previous study of COBALT, that D10, a microtubule-binding agent, particularly enhanced the tumoricidal ability of *C. novyi*-NT more than other classes of chemotherapeutic agents, Dang *et al.* used microtubule stabilizers and microtubule destabilizers to combine with *C. novyi*-NT spores treatment, respectively. The results suggested that the latter, microtubule destabilizers not only inhibited the tumor growth by targeting tumor vasculatures, but also decreased blood flow and increased the extent of hypoxia within tumors as well, thereby creating a larger area for *C. novyi*-NT spores to germinate and multiply. Most importantly, the lethal toxicity of COBALT was successfully controlled to a quite acceptable degree, while the high cure rate was still guaranteed [31,47]. In 2005, it was reported that (+)-2,3-anhydrodiscodermolide, an analogue of (+)-discodermolide, which retains activity against paclitaxel-resistant cell lines, was used as the microtubule-stabilizing drug in COBALT. The use of (+)2,3-anhydrodiscodermolide and *C. novyi*-NT spores successfully led to massive hemorrhagic necrosis and complete tumor regression in all mice; four of five mice were apparently cured [48].

As a conventional therapeutic method of cancer treatment, radiotherapy obviously seemed worth a trial in combination with *C. novyi*-NT spores because theoretically, the properties of *C. novyi*-NT compensated for tumor resistance to radiotherapy resulting from low

level of oxygenation within the tumor [29,49]. In 2003, Dang *et al.* selected three modes of radiation to combine with *C. novyi*-NT treatment to test this hypothesis. The results showed that *C. novyi*-NT can enhance the efficiency of radiotherapy. In addition, different forms of radiation in combination have their own merits. When *C. novyi*-NT was used in combination with brachytherapy, a 100% cure rate of mice bearing HCT116 and HuCC-T1 xenografts was observed. With external beam radiation, *C. novyi*-NT treatment significantly decreased the dose of radiation, in contrast to using radiation alone, without the limitation of tumor volume. Therefore, therapy at sites in which more conventional doses of radiation are toxic, such as liver, became quite amenable. Similarly, toxicity to normal tissues induced by RAIT was minimized because of the inclusion of *C. novyi*-NT [30].

Targeting cancer cells without harming healthy ones is becoming a vital focus of cancer treatment. As we have mentioned above, conventional options by increasing dose of chemotherapy or radiotherapy have been proved unfeasible because of the simultaneous increased toxicity to healthy organs. In order to reduce systemic toxicity, scientists proposed liposome to encapsulate chemotherapeutics [50]. After years of trials and improvements, sterically stabilized liposomes (SSLs) stood out gradually and became priority [51]. Unfortunately, although we have reduced the toxicity to normal organs by using SSLs to encapsulate 'double-sword' chemotherapeutics, we still seemed helpless when facing drug's low rate of degradation within tumor, which caused prolonged time of therapy [52,53].

As well documented, *C. novyi*-NT is a hemolytic bacterium because some of its enzymes that lyze erythrocytes can disrupt lipid membrane [54]. We have seen the potential of *C. novyi*-NT spores as tumoricidal agents and its characters of anaerobic germination and multiplication [29]. On the basis of this, *Cheong et al.* [33] hypothesized that *C. novyi*-NT promotes the release of chemotherapeutics from SSLs within tumor by lyzing the liposome. To test this hypothesis, *Cheong et al.* carried out the experiments of treating mice bearing established tumor with *C. novyi*-NT spores and liposomal doxorubicin (Doxil), which is a clinically common liposomal cancer drug. As a result, they observed that 100% of mice displayed complete tumor regression and 65% of them survived the whole experiments in comparison to unsatisfactory results of control models, which were treated with *C. novyi*-NT spores or Doxil alone. Apart from this, all mice treated with *C. novyi*-NT and free doxorubicin died within 2 weeks, which highlighted again the vital role of liposomal encapsulation in reducing systemic toxicity. The liposomal-disrupting factor, encoded by the NT01CX2047 gene [38], was finally purified and identified as a new lipase (termed liposomase), which would be highly expressed in tumor after injection with *C. novyi*-NT according to subsequent research. Unexpectedly, the liposome-disrupting activity of liposomase was identified as physical process – the interaction of its lipid-binding domain with the liposomal membrane and consequent alteration of bilayer structure, suggesting a novel mechanism specific to *C. novyi*-NT [32,33].

Hypoxic cancer cells usually possess the properties of invasion and metastasis as well as resistance to chemotherapy and radiation therapy. Based on the therapeutic strategy of diminishing hypoxia, hypoxic-inducible factor alpha (HIF-1α), which is the key regulator of hypoxia, has emerged as a promising target [1,12,44,45]. Although highly specific antibodies targeting HIF-1α show more advantages over common cytotoxic agents, their delivery is hampered by poor vasculature within solid tumor; thus, the lethal hypoxic cancer cells could be spared. Groot *et al.* [35] proposed a new therapeutic method, namely *Clostridium*-directed antibody therapy, that certain *Clostridium* are incarnated to vectors and produce antibodies specific for tumor antigens. In 2007, VHH (variable domain of the heavy-chain antibody), a small, specific antigen-binding protein, which can inhibit HIF-1α activity when binding to HIF-1α, and stimulating agent that will enhance the expressing level of *VHH* gene, were introduced into *C. novyi*-NT by heterologous gene transfer. After successful conjugation and expression, the VHH generated by *C. novyi*-NT vectors was proved functional because it retained its binding capacity and specificity for HIF-1α antigen. These trials demonstrated that *C. novyi*-NT could be further modified to function more efficiently and effectively.

Safety concerns

To ascertain the safety of spores of *C. novyi*-NT as a promising therapeutic, their potential toxicity and pharmacological characteristics were evaluated [29,39,55]. The results illuminated that no signs of clinical toxicity were observed in both healthy and tumor-bearing mice, rabbits, and dogs; in addition, clearance of the spores from circulation happened quickly after systemic injection. Interestingly, spores of *C. novyi*-NT seemingly showed high affinity and specificity to hypoxic regions within tumor because other hypoxic regions, such as myocardial infarcted tissues, were quite clean [55]. The mechanism underlying this phenomenon is still unknown. As for tumor-bearing animals, germination of *C. novyi*-NT caused inflammatory response, thus inducing toxicity, which could be effectively diminished by systemic hydration [55]. With results in hand, the safety study of *C. novyi*-NT moved to a phase I clinical trial in 2006, and the second attempt started in 2010.

Perspectives and challenges

This discussion of several applications of *C. novyi*-NT in antitumor therapy has indicated both its advantages and disadvantages [15,56]. For future *C. novyi*-NT therapy, one rule is unbreakable, namely, successful cancer therapy must be efficient, effective, and safe. Nowadays, although conventional cancer therapies are still dominant, *C. novyi*-NT's high specificity and oncolytic capabilities suggest that more combinational trials are worth trying, which may possibly overcome the disadvantages of either application. In addition, gene therapy is a forward-looking alternative. Cytokines, such as interleukin-2 and tumor necrosis factor-α, highly specific antibodies, targeting tumor antigens, as well as enzymes, converting a prodrug into its cytotoxic form, can be directed into solid tumor by *C. novyi*-NT vector. With the complete *C. novyi*-NT genome sequence available, new promoters that will help increase the level of transferred gene expression could be explored, and in addition to this, tumoricidal proteins, such as lipomase, could be identified more easily as well.

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Conflicts of interest

There are no conflicts of interest.

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