



# Evolution of oncolytic viruses

Rafael Sanjuán<sup>1</sup> and Valery Z Grdzlishvili<sup>2</sup>

Owing to their replicative capacity, oncolytic viruses (OVs) can evolve under the action of natural selection. Reversion to virulence and recombination with wild-type strains may compromise OV safety, therefore requiring evolutionary risk assessment studies. On the other hand, evolution can be directed in the laboratory to create more potent and safer OVs. Previous work in the experimental evolution field provides a background for OV directed evolution, and has identified interesting exploitable features. While genetic engineering has greatly advanced the field of oncolytic virotherapy, this approach is sometimes curtailed by the complexity and diversity of virus–host interactions. Directed evolution provides an alternative approach that may help to obtain new OVs without prejudice toward the underlying molecular mechanisms involved.

## Addresses

<sup>1</sup> Instituto Cavanilles de Biodiversidad y Biología Evolutiva and Departament de Genètica, Universitat de València, Valencia, Spain

<sup>2</sup> Department of Biological Sciences, University of North Carolina at Charlotte, Charlotte, NC, USA

Corresponding author: Sanjuán, Rafael ([rafael.sanjuan@uv.es](mailto:rafael.sanjuan@uv.es))

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## Introduction

Oncolytic virus (OV) therapy uses replication-competent viruses to selectively target malignant cells, in contrast to virus-based gene therapy which uses replication-deficient viruses. Despite a large number of preclinical studies with impressive results that have led to several OVs being currently tested in human clinical trials, some critical challenges need to be addressed, including insufficient selectivity for tumoral cells, low oncolytic potency, inability to penetrate and spread in tumor tissues, premature viral clearance, and poor stimulation of tumor-specific immunity. Many efforts have addressed these issues. Due to the increasing availability of techniques for the genetic manipulation of animal viruses during the last decades, today the field is dominated by an engineer's view based on the rational modification of viral genomes. Hundreds of

genetically engineered OVs have been created in which virulence genes have been deleted; the viral tropism or the ability to escape premature neutralization has been reset by modifying viral envelope proteins; and viruses have been armed with tumor suppressor or suicide genes expressed only in cancer cells, with immunostimulatory genes, or with genes that increase susceptibility of infected cells to chemo and radiotherapy [1–3].

These approaches have undeniably led to important advances, but have also met some difficulties. Virus–host interactions are extremely complex and often still poorly understood, and therefore our ability to manipulate them is limited. This is particularly challenging within the context of virus–tumor cell interactions, as tumor cells vary widely depending on the cancer type and between and within patients [4]. As a result, the rational design of OVs for each specific tumor becomes a formidable task. Additionally, extensive manipulation of viruses often renders them so attenuated, that, in many cases, it abrogates replication. Although strong attenuation is desirable for safety, many OVs have proven to be insufficiently potent against tumors. A unique feature of OV, though, is that, unlike other therapeutic agents, they replicate and mutate, and therefore have the potential to evolve. This has two important implications. First, OVs are amenable to optimization by directed evolution. This approach can help improve the efficacy of previously engineered viruses, and may allow creation of new OVs by acting on still uncharacterized molecular pathways. Second, evolution of therapeutic viruses *in vivo*, once delivered to patients, needs to be seriously assessed and, particularly, the probability of reversion to virulence.

## Directed evolution to generate more effective OVs

Directed and experimental evolution has been used for practical applications in various research areas. For instance, pioneer procedures for creating oral polio vaccines included serial passages in non-human hosts [5]. In light of today's knowledge, this probably favored adaptation to the alternate hosts at the cost of reduced fitness in humans and promoted the accumulation of deleterious mutations by random genetic drift, thus making these basic evolutionary processes instrumental in the success of polio vaccines. Evolutionary studies have also been used to predict the appearance of drug resistances in HIV-1 [6], or the pathogenesis and pandemic potential of influenza viruses [7].

Using modern experimental evolution techniques, it is possible to adapt viruses to cells showing the hallmarks of

cancer, to characterize the genetic and molecular basis of this adaptation, and to subsequently evaluate the usefulness of these OV's in cell cultures and animal models (Figure 1). However, this approach has not been systematically applied in the field, albeit a few notable exceptions [8,9<sup>••</sup>,10,11,12<sup>••</sup>,13,14<sup>•</sup>]. In one study, pools of different adenovirus serotypes were serially passaged in cultures of human colon cancer cells, favoring among-serotype recombination and the selection of the fittest variants in these cells [9<sup>••</sup>]. This led to isolation of a recombinant virus (ColoAd1) that outperformed existing oncolytic adenoviruses and was approved for phase I/II clinical trials. A limitation of using directed evolution with double-stranded DNA viruses, though, is their low rates of spontaneous mutations compared to RNA viruses [15] thus making the production of new potentially adaptive variants slower. However, DNA virus variability can be enhanced using chemical mutagenesis [13] or by engineering viral polymerases with reduced replication fidelity [10].

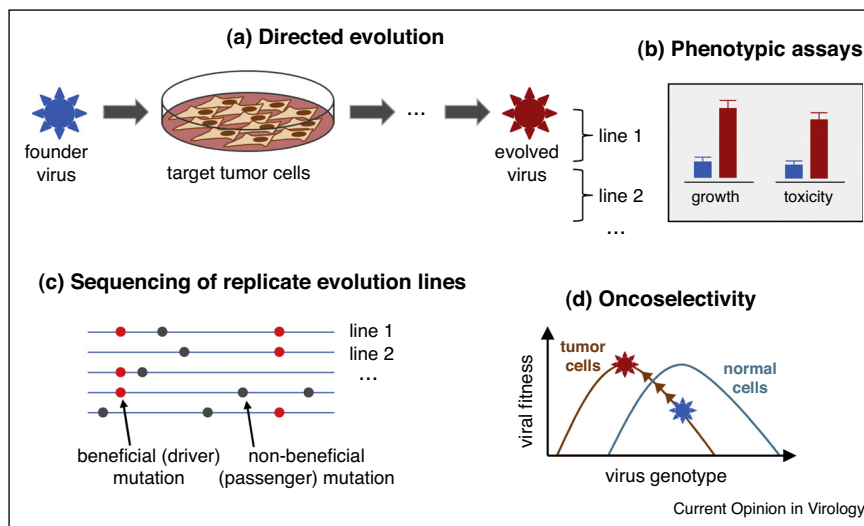
There are also a few examples of directed evolution of RNA viruses in the context of oncolytic virotherapy. In one study, a pseudotyped vesicular stomatitis virus (VSV) was engineered to express a single-chain antibody against the Her2/neu receptor (ErbB2). Although the engineered virus initially showed a very low titer in target mammary cancer cells expressing ErbB2, serial passages in these

cells increased viral fitness [8]. Another example is serial passages of wild-type VSV in human glioblastoma cells with the aim of promoting selective attachment to these cells and replication [11]. Interestingly, the evolved virus was later found to be effective also against other tumor cell lines [12<sup>••</sup>]. Recently, VSV was adapted to MEF p53<sup>-/-</sup> cells by serial passaging and then tested in isogenic p53<sup>+/+</sup> cells, as well as in p53-positive and p53-negative tumors *in vivo* [14<sup>•</sup>]. This revealed gene-specific adaptation, suggesting that VSV can be selectively adapted to a broad cancer feature such as p53 inactivation.

### Relevant factors in directed evolution experiments

Owing to their extremely high rates of spontaneous mutation, RNA viruses are ideal candidates for directed evolution. This, combined with the often high titers achieved under cell culture conditions, increases the efficacy of selection and allows for deterministic evolution of fitness-related traits in the laboratory [16]. Additionally, the genomes of most RNA viruses are less than 20 kb, making it easy to identify mutations responsible for adaptation. However, with the advent of next-generation sequencing, the genetic analysis of large oncolytic DNA viruses such as vaccinia or herpes viruses has been greatly facilitated, allowing direct identification of the molecular basis of adaptation in these viruses as well [17]. Using

Figure 1



Directed evolution of oncolytic viruses. **(a)** Starting from a founder virus, which can be the wild-type or a previously engineered virus which has to be optimized, serial transfers can be performed in target tumor cells under conditions that favor the action of selection (high effective population size, low multiplicity of infection). The duration of this process can vary depending on the type of virus (RNA/DNA) and the strength of the selective pressure, but should typically range 10–50 transfers. The repeatability of evolution can be assessed by establishing several replicate lines. **(b)** The growth rate, toxicity, or other interesting phenotypic properties can then be evaluated for the evolved virus and compared against the founder. **(c)** Sequencing of replicate evolution lines can help identify relevant mutations responsible for the observed increases in viral fitness (driver mutations), since these mutations typically appear in more than one line (parallel evolution). **(d)** In many cases, adaptation to a specific cell type (here a given tumor cell line) is accompanied by a loss of fitness in other cell types (here, normal cells). These fitness tradeoffs should increase the oncolytic selectivity of the evolved viruses.

next-generation sequencing, it is also possible to study the genetic variation of viral populations in great depth [18]. In some cases, this variability is a key determinant of viral fitness [19]. Therefore, identification of mutations that became fixed in the consensus sequence of a viral population may provide only limited information about the genetic basis of adaptation.

Previous experimental evolution studies with RNA viruses and small DNA viruses resulted in two general observations that are particularly relevant for the directed evolution of OV. First, the same substitutions often appear repeatedly in independently evolving lines, a process called parallel evolution [20–23]. Therefore, by performing several replicates of a directed evolution experiment, it is possible to differentiate ‘driver’ mutations responsible for the observed desired traits (increased fitness/toxicity in tumor cells) from ‘passenger’ mutations which have increased their frequency by genetic linkage to another, selected mutation (genetic hitchhiking) or become fixed in the viral population by random genetic drift (Figure 1c). Second, selectively advantageous mutations in one environment are often costly in alternate environments in which the virus was not evolved [21,24–26]. These fitness tradeoffs are particularly common in RNA viruses, where most spontaneous mutations are strongly deleterious [27], and because many viral proteins are multifunctional. Therefore, evolutionary optimization in one environment tends to produce de-optimization in alternate environments, especially when these environments are markedly distinct (Figure 1d). Ecological theory predicts that these fitness tradeoffs should favor specialization [28]. Therefore, serial passages in a given tumor cell type is expected to produce specialized viruses with reduced fitness in other cell types, including normal cells.

Although the general conceptual scheme of a directed evolution experiment is very simple, there are key variables that need to be carefully considered. A major determinant of the rate of adaptation is the effective population size, which can be easily manipulated in the laboratory by varying a multiplicity of infection (the number of infectious virus particles per cell). If the population size is small, genetic drift will prevail over selection and there will be little or no adaptation [29]. If, on the other hand, the population size is very large such that the multiplicity of infection exceeds one virus per cell, adaptation can be hampered because low-fitness variants can benefit from genetic complementation in cells infected by multiple particles [30]. This promotes the evolution of hyperparasites, the extreme case being defective interfering particles [31].

Other factors to be considered include the number of passages, the number of evolution replicates, and the type of selective pressure used for directing evolution. For

fast-mutating viruses such as RNA viruses, a few passages may suffice to achieve significant adaptation, while further passages may essentially lead to neutral divergence and complicate identification of relevant adaptive mutations. Having a sufficiently large number of replicate evolution lines (e.g., five) greatly simplifies this task by helping to identify parallel substitutions. Finally, for most viruses, tumor cells constitute a naturally permissive environment in which some loss-of-function mutations are tolerated, such as loss of interferon (IFN) blocking. As a result, tumor-specific replication would not be directly driven by positive selection of new variants, but, instead, by a relaxation of selection in tumor cells [14<sup>\*</sup>], and there should be little parallel evolution in these cases.

### Potential risks associated with evolution of OVs

There are practically no studies addressing potential risks associated with OV evolution. Some candidate OVs approved for phase I/II clinical trials have been constructed by cloning human IFN genes in wild-type virus genetic backgrounds (e.g., trial NCT01628640) [32,33]. Although these viruses were shown to be safe due to their compromised abilities to replicate in healthy tissues, IFN severely reduces viral fitness in healthy tissues, making it highly likely that this type of cassette undergoes partial or full deletion of IFN coding sequences after few rounds of replication. A similar outcome is expected for OVs encoding tumor-suppressor genes with known antiviral effects, such as p53 [34] which inhibits viruses via stimulation of type I IFN antiviral signaling and apoptosis [35]. Furthermore, OVs are often administered in combination with mutagenic chemotherapeutics, which may increase the viral mutation rate and enhance evolution, particularly in DNA viruses.

In addition to back mutation, recombination may occur between OVs and other, closely related viruses co-infecting the patient. This is especially a risk for viruses with high prevalence and elevated rates of recombination, such as adenoviruses. Also, novel recombinant OVs are often generated by introducing heterologous viral genes into the genome. For example, a recently described recombinant herpes simplex virus (HSV) carries the vaccinia virus B18R gene, which encodes a secreted decoy receptor with a broad antagonizing effect against type-I IFNs [36]. While the evolutionary potential of these artificial recombinants has not been assessed, in principle, these heterologous genes may be transferred to wild-type genetic backgrounds and create potentially harmful viruses (e.g., wild-type HSV with the B18R gene). Finally, several screenings of wild-type animal viruses have been conducted to identify new OVs [37<sup>\*</sup>]. Although these studies produced very promising OV candidates, there is very limited information about their tropism and virulence determinants, and hence the risk that these viruses may evolve into emerging human pathogens is unknown.

Therefore, evolutionary risk assessment studies should be performed to ensure that reversion to virulence, recombination with wild-type strains, and gain of function mutations leading to adaptation to humans can be ruled out.

## Conclusions

OVs replicate, mutate, and have low fitness because they have been genetically modified to make them attenuated. Therefore, OVs should rapidly evolve under the action of natural selection. Directed evolution offers a useful tool to increase the fitness and oncoselectivity of previously engineered OVs, and to overcome some of the limitations imposed by the complexity of virus–host interactions. Previous work in the field of experimental evolution has revealed some interesting features. Parallel molecular evolution is widespread in RNA and some DNA viruses and, therefore, use of replicate evolution lines can facilitate the identification of candidate adaptive mutations. However, in some cases, phenotypic changes may not be accompanied by the fixation of new mutations in the viral population, thus requiring an in-depth analysis of variability using massive parallel sequencing. Also, for many viruses, adaptation to a given cellular environment often comes at the cost of lowering fitness in alternate environments, thus promoting ecological specialization, a feature that could be exploited to enhance the tumor-specificity. In order to successfully direct evolution toward cancer target cells, the effective population size, the multiplicity of infection, the rate of spontaneous mutation, the number of evolution replicates, the number of serial passages, and the selective forces at play are key factors that should be considered. Finally, experimental evolution can also help us to assess the risk of reversion to virulent states during treatment, thus providing increased safety.

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