

REVIEW

Gene therapy as a potential tool for treating neuroblastoma—a focused review

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Neuroblastoma, a solid tumor caused by rapid division of undifferentiated neuroblasts, is the most common childhood malignancy affecting children aged < 5 years. Several approaches and strategies developed and tested to cure neuroblastoma have met with limited success due to different reasons. Many oncogenes are deregulated during the onset and development of neuroblastoma and thus offer an opportunity to circumvent this disease if the expression of these genes is restored to normalcy. Gene therapy is a powerful tool with the potential to inhibit the deleterious effects of oncogenes by inserting corrected/normal genes into the genome. Both viral and non-viral vector-based gene therapies have been developed and adopted to deliver the target genes into neuroblastoma cells. These attempts have given hope to bringing in a new regime of treatment against neuroblastoma. A few gene-therapy-based treatment strategies have been tested in limited clinical trials yielding some positive results. This mini review is an attempt to provide an overview of the available options of gene therapy to treat neuroblastoma.

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INTRODUCTION

Cancer, the uncontrolled proliferation of cells, has become a scourge and a major concern of human health. Several efforts have been made to design and develop treatment regimens to this health menace. However, with astounding complexity both at phenotypic and genetic level, cancer shows great diversity in therapeutic resistance.¹ Some of the long standing cancer treatments include surgery, chemotherapy, radiation, hormone therapy and differentiation therapy. The major challenge faced by all of the above approaches is the inability to distinguish a cancerous cell from a benign or normal cell. Due to this there are many side effects of these anti-cancer treatments resulting in poor outcome and a lot of discomfort to patients.¹ Of the many approaches practiced and the treatment options available, gene therapy using both viral and non-viral vectors is fast gaining ground.² Gene therapy in its early days was opined, largely due to the technical limits of the delivery mechanisms, to be applicable only in cases of correcting the single gene defects that result in hereditary diseases.³ However, over the last few decades, cancer has established itself to be a clear case for gene therapy. At present both viral and non-viral methods of gene delivery are used to attempt treatment for many diseases including cancer.⁴

Neuroblastoma, the most common solid tumor in children below the age of 5, is a malignancy caused by hyperplasia of naive neural crest cells.⁵ Accepted treatments of neuroblastoma include intensive chemotherapy, removal of primary tumor by surgery followed by high-dose chemotherapy and autologous hematopoietic stem cell rescue.⁵ However, even with intensive therapy, a variety of challenges limit the average long-term life expectancy and survival of a diagnosed patient (less than 20%) and thus offer poor prognosis for neuroblastoma.⁶ Hence, we need to consider gene therapy as an option for neuroblastoma treatment (Figure 1).

Many of the key genes that regulate progression of the tumor are not only involved in cell-cycle progression, but also in other important cellular functions like glucose metabolism and lipid synthesis. For example, many of the key genes are known to be activated by one of the major oncogenes called *myc*. The *myc*-binding regions are randomly distributed throughout the entire human genome (> 10 000 *myc*-binding regions distributed throughout the genome). Of the many challenges faced by the amplification and over expression of normal *myc* genes, the diversity seen in this gene remains one of the major reasons for high-risk tumor transgression and poor prognosis.⁷ *Myc* is known to be mutated in many cancer types and therefore could be a good target for gene therapy as a therapeutic approach to counter cancer.⁸ In this mini review, we aim to provide an overview of the different viral and non-viral vectors along with their target genes used to treat neuroblastoma and also critically review the results thus obtained to delineate the drawbacks and limitations of each system.

VIRAL METHODS

The success of a gene therapy depends on development of efficient targeting systems. Viral vectors have been extensively used for cancer gene therapy because of their relatively high efficacy of gene transfer with distinct killing mechanisms² (Figures 1 and 2). Retroviral and adenoviral vectors are among the most frequently chosen vector systems.^{9,10} Though more and more efficient delivery systems are being developed, some of the challenges that need to be circumvented in making virus-based vectors as an ideal delivery system include immunogenicity, limited transfer efficacy, stability, level of gene expression and lack of tumor specificity.¹¹

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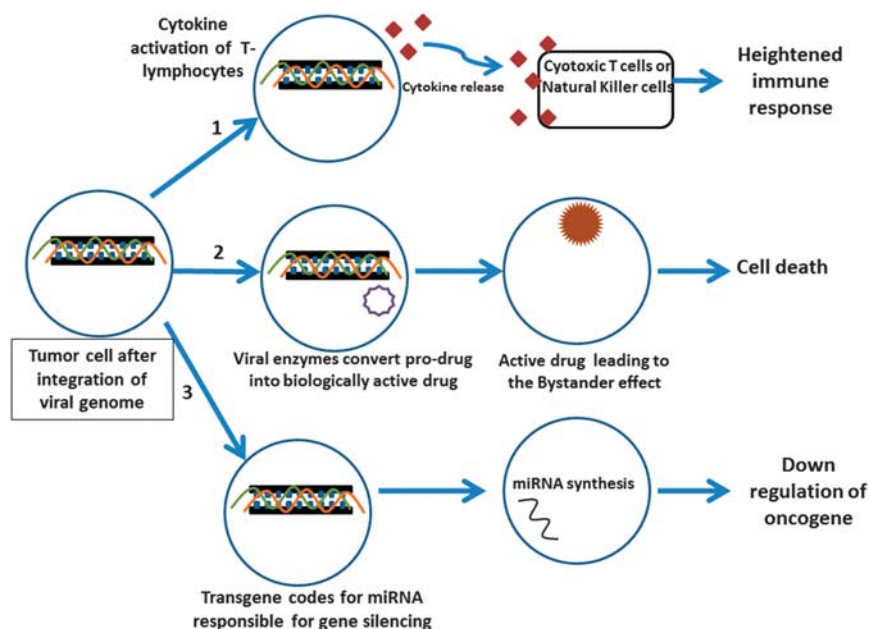


Figure 1. Certain cell death mechanisms that have proven to be effective in neuroblastoma. (1) Immunotherapy: transgene in carrier construct codes for cytokines that activate the cytotoxic activity of T cells. Activated T cells hence eliminate tumor cells. (2) Enzyme-pro-drug therapy: a viral enzyme that has ability to convert harmless pro-drug into cytotoxic drug is introduced into a cancer cell. The pro-drug is then separately added at the site. The drug after enzymatic modification of pro-drug induces programmed cell death. (3) Downregulation via gene silencing: microRNA is a non-coding RNA that is involved in control of gene expression at the transcriptomic level. This concept is exploited to downregulate target oncogene, so that cell initially destined to be a cancer cell will now become a normal cell. ● Active drug, ○ prodrug, ◆ released cytokines, 〰 viral vector genome.

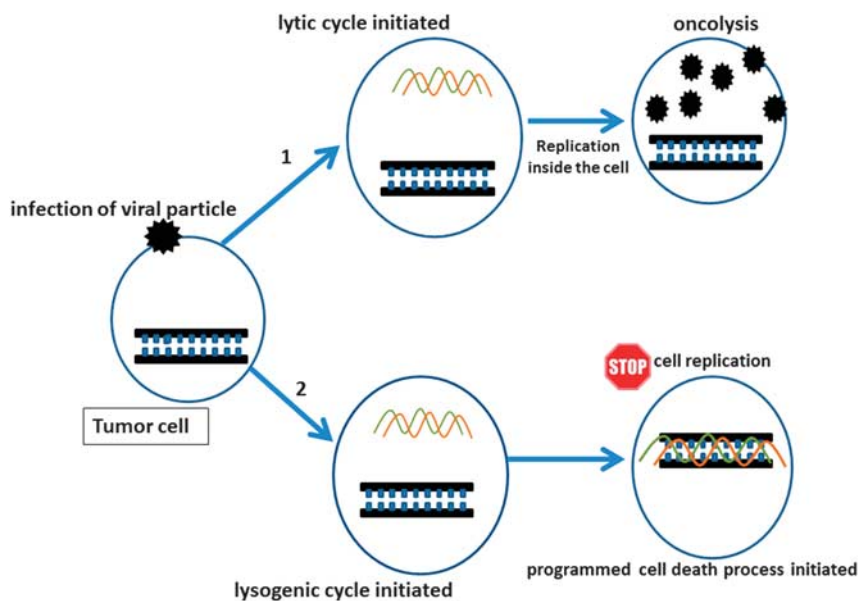


Figure 2. Two strategies for using gene therapy for cancer studies. (1) Direct method: cell-type-specific introduction of viral genome of a lytic virus will eliminate cancer cells. (2) Indirect method: transgene codes for proteins that evoke cell's natural death-inducing mechanisms (like apoptosis, phagocytosis by natural killer cells and macrophages). ● Viral vector, 〰 viral vector genome.

Retrovirus

Retroviral vectors have been crucial to the rise of gene therapy. One of their most distinct advantages is their ability to stably integrate into the host genome which enables a permanent gene transfer. They were first used as gene therapy vectors in the 1990s

against severely compromised immunodeficiency (SCID). However, 3 years post treatment, two of the youngest patients developed uncontrollable proliferation of T cells.¹² After multiple genomic alterations, there were two major successes in the early 2000s that showed regression of symptoms of SCID-X1 and

Adenine deaminase deficiency.^{13,14} By the year 2015, retrovirus has been used as a delivery system to transfer different target genes and in ~18.4% of all clinical trials for gene therapy worldwide (<http://www.wiley.com/legacy/wileychi/genmed/clinical/>).

Retroviruses can work in conjunction with the immune system to provide immunotherapy solutions (Figure 1).^{15,16} The linear correlation between the strength of promoter and level of transgene expression has allowed for engineered forms of retrovirus to be developed having increased anti-tumor activity (Table 1). Engineered retroviruses that express chemokines hijack the cell's protein synthesis machinery after integrating into the genome, so that the immune system can eradicate tumors. CD4+ and CD8+ T cells increase the potential of cancer immunotherapy, with CD8+ T cells being the more attractive option between the two because of direct lysis of cancer cells on recognition of MHC I-peptide complex.¹⁷ One of the methods of launching a potent anti-cancer immune response is the transfer of autologous T cells (that are made reactive to tumor antigens *in vitro*) back into the body intratumorally.¹⁸ In an isolated study an engineered retrovirus expressing interleukin (IL)-2 was transduced to Neuro-2a cells. A murine model of neuroblastoma when injected with these transduced cells did not show tumor growth.¹⁹ IL-2 was chosen because it plays a role in differentiation of CD8+ and CD4+ to effector cells, and maintains balance of their population.²⁰ As a conclusion, the success of this study depended on cytotoxicity mediated by both CD8+ and CD4+ cells.¹⁹ Also, both cell types yielded a similar anti-cancer effect when there was a simultaneous adoptive transfer of IL-2.²¹ However, the establishment of tumor-reactive T cells can be a challenge because cancer antigens are still very 'humanized' to cause spontaneous activation of such cells.²² Thus as seen in the above case the anti-tumor activity by the immune system is time consuming and is established only after the therapy is complete.

Non-toxic pro-drugs can be metabolized by cellular enzymes to ultimately give a biologically active drug.²³ Viral thymidine kinase (tk) converts ganciclovir pro-drug into its tri-phosphate derivative, which is lethal to the cell. This method induces apoptosis in death-receptor and p53-independent and mitochondrial-dependant-Bcl2-modulated pathway, although the mechanism remains controversial.²⁴ In a comparative study to choose the best promoter for this viral enzyme, promoter of tyrosine hydroxylase was identified as the choice promoter for achieving maximum neuroblastoma cell-specific expression of tk²⁵ (Figure 1). The human genome codes for certain non-coding RNAs that are transcribed from the DNA but are not translated to protein. These functional RNA molecules have a major role to play in gene regulation.²⁶ Short Hairpin RNA (shRNA) is an example of such non-coding RNA. It has been observed that expression levels of oncogenes can be down regulated at the protein level using shRNAs. A lentivirus driven *myc*-targeted shRNA introduction into the genome caused a stable downregulation of the oncogene.²⁷ In addition, levels of anti-apoptotic signals also dipped, coupled with an increase in caspase-3 activation and p27 upregulation.²⁷ Thus, while extensive studies have proven the utility of retroviruses in gene therapy approaches to treat neuroblastoma there are still concerns about the mechanisms of action of this form of viral gene therapy (Table 1). In the absence of concrete evidence or quantifiable results there is limited hope that this therapy will lead to a complete remission.

Adeno-associated virus

Adeno-associated virus (AAV) is widely and rapidly gaining popularity as a gene therapy vector mainly because of its non-pathogenicity in humans. Although the virus is present across tissues of various animal species, it is not known to cause any disease.^{28,29} In addition, AAV has low immunogenicity and is able to transduce both non-dividing and dividing cells making it a

viable option in treating neuroblastoma.^{30–32} Another advantage of AAV as a gene therapy vector is its unique ability for sustained expression especially in case of neuroblastoma thereby establishing a practical method for long-term delivery of the therapeutic genes (Table 1).³⁷ AAV also has unique properties where stable transgene expression can be achieved with no effect on the normal angiogenesis processes. This aids in the use of this vector in targeting aggressive tumors with metastatic properties (Table 1). About 6% of all approved gene therapy trials use AAV as the vector (<http://www.wiley.com/legacy/wileychi/genmed/clinical/>).

Vascular endothelial growth factor (VEGF) is a protein/chemokine expressed by oxygen-stressed, hypoxic cells, so as to initiate angiogenesis.³³ However, after a certain stage, the oxygen levels at the center of the tumor reduce leading to upregulation of VEGF, which is mediated by hypoxia-inducing factor (HIF)-1 α .^{34,35} According to a set of evidences, there was an inverse correlation between the expression of VEGF and VEGF receptor-2 (VEGFR2) in tumor cells.³⁶ That is to say, VEGFR2 expression can have a negative effect on the expression of the major pro-angiogenic protein, VEGF. A study engineered a long-term, recombinant AAV-expressing VEGFR2 (a decoy receptor for VEGF) to target the endothelial cells and showed improved survival of mice affected with disseminated neuroblastoma.³⁷ VEGFR2 binds to VEGF with high affinity, and would dominate decisions regarding vascular permeability and endothelial proliferation.³⁷ Cytokines like interferons (IFNs) and ILs play a versatile role in cell-cell communication. IFNs show anti-tumor activity by preventing differentiation of endothelial cells.³⁸ Many IFN stimulated genes are known to be responsible for the generation and proliferation of cytokines essential for inhibition of angiogenesis which is one of the primary reasons for tumor spread.³⁹ AAV-mediated, liver-directed stable expression of IFN- β was shown to cause regression of neuroblastoma size, mainly due to lowering of intra-tumoral vascular tissue density.⁴⁰ Combination with chemotherapy only adds to the success, in which there is a complete removal of cancerous tissue. AAV-mediated delivery of IL-2 in combination with the chemokine Fractalkine has also been shown to help increase anti-tumor efficacy.⁴¹ Although AAV has not shown successful results in human trials for neuroblastoma, it is still one of the most malleable viral vectors to work with considering its safety profile. However, it remains as a viable gene therapy option only in the presence of a combination therapy [37] [40]. The inability of this virus to cause tumor regression in isolation has been a major cause of concern in the studies moving from mouse models to human trials (Table 1).

Adenovirus

Adenovirus (Ad) is known to be the most widely used vector for gene therapy and now has been used to show extensive progress even in the case of neuroblastoma. Replication defective Ad vectors are used as gene delivery vehicles and vaccines, whereas replication-competent (oncolytic) vectors are employed for cancer gene therapy (Figure 2).¹⁰ The term oncolytic virus refers to the ability of a virus (inherent or genetically engineered) to specifically recognize tumor cells and subsequently kill them.⁴² Ads are known to be non-pathogenic systemically while ensuring long replication cycles. They also induce differentiation of neuroblasts while inhibiting cell-cycle process (Table 1). Several attempts that have been made to use Ad as a delivery system in gene therapy against neuroblastoma are discussed here.

One of the early studies includes the use of Ad to transfer IL10 into autologous unirradiated tumor cells and then using these cells to enhance the immune response against the existing tumor.⁴³ The therapy which was tested in 10 children with advanced neuroblastoma was found to enhance anti-tumor activity mediated by IgG antibodies and increased cytotoxic T-cell killing of the tumor. There was also an increase in local, as well as

Table 1. Comparison of the different viral vectors used in neuroblastoma treatment

	<i>Ad</i>	<i>AAV</i>	<i>HSV</i>	<i>Retrovirus</i>	<i>Polio virus</i>	<i>Sendai virus</i>	<i>VSV</i>	<i>NDV</i>	<i>Measles</i>
Genome	dsDNA	ssDNA	dsDNA	ssRNA	(+)ssRNA	(-)ssRNA	(-)ssRNA	(-)ssRNA	(-)ssRNA
Capsid	Icosahedral	Icosahedral	Icosahedral	Icosahedral	Icosahedral	Helical ⁹²	Helical ⁹³	Elliptical	Helical
Coat	Naked	Naked	Enveloped	Enveloped	Non-enveloped	Enveloped	Enveloped	Enveloped	Enveloped
Virion diameter	70–90 nm	18–26 nm	150–200 nm	80–130 nm	29 ± 1 nm	110–540 nm	180 nm	100–500 nm in diameter.	50–510 nm
Genome size	~ 36 kb	~ 5 kb	~ 152 kb	7–10 kb	7.4 kb	13.5 kb	180 nm long, 75 nm wide	15.1 kb	15.9 kb
Disease causing in humans	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes
Transgene expression	Transient	Potentially long lasting	Potentially long lasting	Long lasting	Long lasting	Transient but very strong expression	Transient	Transient	Transient
Maximum packaging capacity	~ 30 kb	~ 4.7 kb	~ 150 kb	~ 8 kb	—	~ 3.2 kb	—	~ 3.8 kb	—
Pros	<ul style="list-style-type: none"> -Maximum data available -Toxicity of virus does not leak out to systemic circulation⁴⁴ -Irradiation of carrier cells does not affect replication capacity⁴⁴ -Very promising; one case of refractory stage IV neuroblastoma showed complete remission⁴⁴ -Long-term replication of virus achievable (up to 3 weeks) -Induces cell-cycle inhibition and causes differentiation of neuroblasts⁴⁷ 	<ul style="list-style-type: none"> -One of the few therapies that target neovascularization and metastasis properties of aggressive tumor³⁷ -Stable transgene expression can be achieved⁴⁰ Does not affect normal angiogenesis processes³⁷ -No immune response generated⁴⁰ 	<ul style="list-style-type: none"> -Replication of HSV not affected by the expression of cytokines (e.g., IL-12)⁵³ 	<ul style="list-style-type: none"> -Cytotoxic effect of the GCV can be transferred to surrounding cells without actual infection with retrovirus²⁵ -Linear correlation between the strength of promoter and level of expression of transgene²⁵ -Anti-tumor immunity is achieved after therapy is completed¹⁹ -Expression levels of oncogene can be reduced at protein level with the help of shRNA²⁷ 	<ul style="list-style-type: none"> -Possible to avoid poliovirus-induced paralysis⁶¹ -It may cause lower damage than chemotherapy and radiation therapy⁶¹ -High rate of mutation in genome helps to increase rate of replication as compared with the initial virus⁶¹ -Existing immunity does not hamper therapy⁶¹ 	<ul style="list-style-type: none"> -Cytotoxic activity can be increased in cells not expressing receptors by adding 13-cis-retinoic acid.⁶² -13cRA pretreatment does not induce killing in normal cells (e.g., skin cells)⁶² -Can stage a immune response in cancer cells derived from xenografts <i>in vivo</i>⁶² 	<ul style="list-style-type: none"> -Tumor cell killing mediated by dual mechanism -GCV induced bystander effect and cytotoxic activity of cytotoxic T lymphocytes⁶³ -Successful elucidation of immune response via IL-4 expression⁶³ -Long-lived expression (up to 16 weeks) possible⁶³ -Minimal immune response against virus is generated⁶³ -High specificity⁶³ 	<ul style="list-style-type: none"> -Efficiency of infection can be increased when added on combination of retinoic acid⁶⁵ -Oral administration possible⁶⁵ -Normal human fibroblasts and athymic mouse are unaffected by the virus⁶⁵ -Following sensitization with retinoic acid, there is rapid replication of virus⁶⁵ 	<ul style="list-style-type: none"> -Localized therapy using Edmonston strain is possible⁶⁴ -Tumor cell-specific delivery of transgene payload is possible with the help of known tumor markers CD46, nectin 4 and other unknown receptors⁶⁴ -Efficient regression of tumors possible, albeit differing kinetics in different cell lines⁶⁴
Cons	<ul style="list-style-type: none"> -Easy clearance by immune system following IV injection -Do not have an independent metastatic targeting ability; needs a carrier cell to transport virus to tumor site⁴⁴ 	<ul style="list-style-type: none"> -Higher vector doses does not translate to higher expression of transgene⁴⁰ -Virus may not be successful in providing the same efficiency it achieved in murine models(murine HSCs) vs human model (hHSCs)³⁷ 	<ul style="list-style-type: none"> -Lack of exact immunocompetent model -Shows temperature dependent replication⁵² 	<ul style="list-style-type: none"> -This strategy does not cause complete remission of cancer -Exact mechanisms of cell death not known 	<ul style="list-style-type: none"> -Replication of these viruses is highly temperature restricted⁶¹ -Remission, in some cases, is not permanent, combination therapy needed⁶¹ 	<ul style="list-style-type: none"> -Remission not permanent.⁶² -Smaller transgene-carrying capacity. 	<ul style="list-style-type: none"> -Longer time required (up to 4 days) to reach maximum effect⁶³ 	<ul style="list-style-type: none"> -Low efficiency of infection independently⁶⁵ 	<ul style="list-style-type: none"> -Precise mechanism how the virus induces tumor cell death after syncytia is not known⁶⁴ -Exact receptors of the Edmonston virus and mechanism of injection in transgenic model is not known⁶⁴

Abbreviations: DS, double stranded; shRNA, short hairpin RNA; SS, single stranded.

systemic inflammatory response mediated by CD4⁺ T/CD25⁺ cells and DR⁺ CD3⁺ T cells, respectively.⁴³

Due to the natural tumor stroma engraftment property of human mesenchymal stem cells (MSCs), these cells easily qualify as cellular vehicles for transport of oncolytic Ad to neuroblastoma site.⁴⁴ Engineered oncolytic Ad can also be delivered with the help of ultrasound guidance.⁴⁵ Both the methods showed decreased tumor size few weeks after introduction of the virus at the tumor site. RISBASEs belong to a family of RNAses that carry specialized biological roles ranging from angiogenesis to host defense.⁴⁶ hPNPase is one such RISBASE. Adenoviral-mediated introduction of hPNPase in human neuroblastoma cell lines SK-N-SH and NGP caused a three to five fold increase in effector caspase (caspase-3, 7) expression, thus inducing apoptosis.⁴⁷ Carboxyl esterase enzyme is involved in detoxification of xenobiotics and activation of ester and amide pro-drug. Adenoviral cloning of rabbit carboxyl esterase in neuroblastoma cell line has a similar mechanism of action as that of Herpes simplex virus (HSV)-tk method: rCE converts the inactive drug CPT-11 to its activated form SN-38. SN-38 is shown to have specificity toward neuroblastoma cells *in vitro*. However, unlike HSV-tk no 'bystander effect' was observed.⁴⁸

Each adenoviral gene has a conserved promoter site, where the transcription begins, and an optional enhancer site to drive home the expression. By shuffling between these two sequences and trying out multiple promoter sequences for desired transgene, the transfection efficiency can be increased.⁴⁹ Particularly in the case of adenovirus, owing to the fact that fiber proteins hamper the binding of Ad to the CAR (Ad receptor), there is lowering of transgene expression. Use of cell-penetrating peptides eliminates the need of CAR, and hence increases the efficiency of transduction.⁴⁹ HIV-1 viral protein R (vpr) is reported to block cell division in G2 phase and induce apoptosis by a multitude of pathways. Intra-tumoral injection of adenovirus with cloned vpr has been shown to reduce tumor size in both, drug resistant and drug sensitive cells.⁵⁰ However concerns regarding clearance of the viral particles by the immune system remain a major barrier to its continued use as a gene therapy vector (Table 1).

Herpes Simplex Viruses

HSV exist in dormant form in the neuronal cells.⁵¹ This fact can be exploited to trigger a site specific cytotoxic activity. HSV is also reported to have a temperature-dependant expression in certain neuroblastoma cell lines (IMR-32).⁵² In another study, the cytotoxic effect of an engineered HSV coding for both the subunits of IL-12 was compared against that of a neuro-attenuated virus having both of its γ_1 34.5 deleted. The median survival of the former was found to be more than that of the latter.⁵³ IL-12 was chosen because it activates the helper-T-cell response, which elicits a more durable anti-tumor activity. In an independent experiment, a multimutated HSV (NV1066) was exposed to eight different cell lines and all of which were found to be sensitive to the engineered vector.⁵⁴ Although the rates of synthesis of viral proteins differed for different cell lines there was evidence of increased survival of animals subjected to an intra-tumoral injection of NV1066 as compared with wild-type Ad type 5.⁵⁴ An important advantage of the HSV vector lies in the fact that replication remains unaffected despite the expression of cytokines such as IL-2 (Table 1). The increase in replication of HSV can also be stimulated by treatment of certain hormones, like synthetic glucocorticoid hormone dexamethosone.⁵⁵ Cytotoxic activity of tk can be increased by incorporating herpes virus surface proteins in mammalian vectors.⁵⁶ A conditionally replicative HSV1 called G207 showed anti-tumor activity without significant toxicity or adverse event in a phase I clinical trial.⁵⁷ Later it was shown that combining G207 with sequential intra-tumoral injections of immature dendritic cells could further improve anti-tumor activity by enhancing

immune response against the tumor in a preclinical model of neuroblastoma.⁵⁸ Hence these extensive trials in both murine and human have successfully established the competency of HSV as a gene therapy vector especially in case of neuroblastoma where the survival rate remains < 50%.⁵⁹

OTHER VIRAL VECTORS

There are other viral vectors used in gene therapy approaches and these are discussed briefly in this section. Polio virus can infect preferentially motor neurons while its high mutation rate ensures sustained replication activity.⁶⁰ A specially engineered oncolytic poliovirus caused complete elimination of neuroblastoma cells after intra-tumoral injection via T-cell mediated immunity. Moreover, these viruses also 'vaccinated' the mouse models against a further exposure to neuroblastoma cells, injected via tail-vein. Polio virus remained non-pathogenic and existing immunity against the virus did not hamper its replication activity. The virus also did not cause paralysis of the host indicating a clear advantage of using this vector.⁶¹ However, the condition-dependent replication of these viral vectors leaves little scope for its use in human trials (Table 1). GD1a is a co-receptor of hemagglutinating virus of Japan-envelope (HVJ-E), a serotype of Sendai virus and co-incidentally is found to be over expressed in the neuroblastoma cell line SK-N-SH. This virus was thus used for oncolytic therapy against neuroblastoma. Combination therapy with cis-retinoic acid increased prognosis and reduced tumor size while sparing normal cells.⁶² Cis-retinoic acid pushes the neuroblastoma cells to differentiation via the RXR-RAR retinoic acid pathway, hence limiting the clonogenicity of the tumor.⁶² However, questions about the mechanism of action remain unanswered. This coupled with its small transgene-carrying capacity does not make this a viable tool for efficient treatment of neuroblastoma. Vesicular stomatitis virus (VSV) can differentiate a defective IFN system from a non-defective one allowing for high specificity to be established in the therapy.⁶³ VSV vectors cause tumor regression by means of both the 'bystander effect' and activity of T-Lymphocytes. This property allows for successful evasion of the immune response via IL-4 expression. Modified VSV coding for IL-2 or tk was shown to reduce tumor growth both in *in vitro* and *in vivo* models.⁶³ Integration of a strain of measles virus (modified edmonston-MV-CEA) was capable of inducing apoptosis in tumor cells of mice that were injected with SK-N-SH cell.⁶⁴ Newcastle disease virus (NDV) showed cell-specific replication in neuroblastoma cells against fibroblasts *in vivo* and *in vitro*. Neuroblastoma cells can be sensitized to increase NDV-mediated cytotoxicity by treatment with differentiating agents like retinoic acid and neuraminidase.⁶⁵ Thus, as discussed above, there are several viral-based therapies that have been attempted to treat neuroblastoma. In these attempts, different genes have been targeted for silencing/overexpression and the attempts have been made using different cell lines or mouse models. These approaches and attempts have met with varied levels of success and thus offer a hope for further exploitation to treat this important type of debilitating brain cancer. Therefore, viral vector-based strategies of gene therapy appear to be a potent tool to treat cancer and a treatment regime is on the horizon. A comparison of general properties of the viral vectors used for neuroblastoma treatment along with their pros and cons is given in Table 1. A summary of the attempts made so far using virus based therapy has been provided in (Table 2).

NON-VIRAL GENE THERAPY STRATEGIES

While treatment for neuroblastoma using viral vectors is gaining rapid traction, it is often noticed that viral methods have lower efficacy levels due to high immune response preventing complete tumor regression.⁶⁴ The classical treatment has proven futile as

Table 2. Summary of the attempts at gene therapy using viral vectors till date in neuroblastoma treatment

Viral vector	Genes targeted	Results	Cell lines/mouse models used	References
Retrovirus	IL-2	Intra-tumoral injection of engineered retrovirus caused regression in tumor size	Neuro-2a cells	19
	Myc-targeting shRNA	Short hairpin RNA delivered by retrovirus caused downregulation of myc gene, coupled with upregulation of p27 and caspase-3	LAN-1 and IMR-32	27
Adeno-associated virus	VEGFR2 (vascular endothelial growth factor receptor)	Upregulation of VEGFR2 resulted in downregulation of VEGF—the most potent factor in tumor angiogenesis.	Mice models:B6.CB17- Prkdc < SCID > SzJ (Jackson Lab, Bar Harbor, ME, USA)	37
	IFN-β	Stable levels of expression of IFN-β caused tumor regression in liver, mainly due to decrease in intra-tumoral vascular tissue	Human neuroblastoma cell lines: IMR-32 and SK-N-AS	40
Adenovirus	Oncolytic therapy	Tumor site-directed transfer of engineered adenovirus caused lysis of tumor cells	Human MSC cultures obtained via bone marrow aspiration	44,45
	hPNPase	Introduction of enzyme showed evidence of upregulation of downstream apoptosis factors, caspases 3 and 7	SK-N-SH and NGP	47
	Carboxyl esterase	Enzyme carboxyl esterase converts inactive pro-drug CPT-11 to its active form, SN-38. SN-38 exhibited cytotoxic activity.	SJNB-1, NB-1691, SK-N-SH	48
Herpes simplex virus	IL-12	The median survival rate of virus coding IL-12 was more than that of another mutant with both the copies of (gamma)-134.5 deleted	Neuro-2a, Human Foreskin Fibroblast (HFF)	94
	Multi-mutated HSV	Eight different neuroblastoma cell lines were found susceptible to the multimutated strain (NV1066), though the levels of sensitivity were different	8 Neuroblastoma cell lines, CHLA-20 and LAN-5	54
	Conditionally mutated HSV-1-G207	Increase in replication of HSV was observed in the presence of synthetic glucocorticoid hormone dexamethosone. Anti-tumor activity and long-term presence of viral DNA, no toxic or adverse effect	Neuroblastoma cells (NB) and Vero cells	55
	Combination of the conditionally mutated HSV-1-G207 and immature dendritic cell	Reduction in tumor volumes, prolonged survival	Phase-I clinical trial	57
Polio virus	Introduction of indigenous cre element	Oncolytic virus caused complete elimination of neuroblastoma cells <i>in vivo</i>	Pre-clinical neuroblastoma model (mouse)	58
				61
Sendai virus		The virus induced lysis of neuroblastoma cells by action of IL-4 and thymidine kinase	Mouse neuroblastoma cell lines: neuro-2aCD155 human cell lines: SK-N-MC, SK-N-SH, and SH-SY5Y	62
Measles virus (Edmonston strain)	MV-receptor CD46	Increased uptake of the engineered virus by tumor cells as they frequently express CD46	SK-N-SH neuroblastoma cell lines	64
Newcastle disease virus	Wild-type strain	NDV in combination with retinoic acid and neuraminidase caused cell-specific viral multiplication in tumor cells	SK-N-SH, MCF7, HUH-6, normal human skin fibroblast cell lines (BJ-1)	65

Abbreviation: shRNA, short hairpin RNA.

tumors continue to exhibit resistance. The rapid blood stream clearance of the viral vectors has vastly reduced the opportunity for complete eradication of the tumors using this mechanism.⁷⁰ Tissue toxicity arising from systemic introduction of the viral vectors is another cause of concern which has led to the development of better systemic delivery of the genes using non-viral vectors.⁷⁰ However, the biggest challenge in case of non-viral methods of delivery is inefficient tumor targeting. Some of the new upcoming non-viral gene therapies exhibit more efficient systemic delivery with low toxicity levels and increasing the rate of event free survival. For example, downregulating Ran (Ras-related nuclear protein) by transferrin shielded silencing (si) RNA has been shown to reduce tumor growth in a preclinical model of neuroblastoma without any significant toxicity.⁶⁶ In a more recent study, siRNA was used to silence the expression of the anti-apoptotic *Bcl2* gene, which is known to be overexpressed in neuroblastoma.⁶⁷ siRNAs were coated with polyethylene glycol-grafted polyethylenimine and were tested for effective delivery into neuroblastoma cells. The coated siRNA was coupled with super-paramagnetic iron oxide nanoparticle to achieve a targeted non-viral vector visible by magnetic resonance imaging. Significant reduction in tumor growth and considerable increase in tumor cell apoptosis was observed in a preclinical murine model of neuroblastoma following administration of the above mentioned siRNA complex.⁶⁷ Overview of some of the other important non-viral treatments attempted specifically in case of neuroblastoma is given below.

Targeted radioiodine therapy following systemic non-viral delivery of *NIS* gene

Experiments conducted in mouse models have shown that the sodium iodide symporter (*NIS*) gene is a novel therapeutic and diagnostic gene for extra thyroidal tumors like neuroblastoma. The use and characterization of the mechanism by which iodine is actively transported across a basolateral membrane of cells has been deciphered long ago.⁶⁸ *NIS* gene is a good candidate for gene therapy because (a) *NIS* is an ordinary human gene and the protein that it expresses is not toxic (b) it does not elicit a significant immune response that can hamper its efficiency (c) produces a desirable 'bystander effect' as it reduces the level of transduction efficiency required for the therapy to be successful and (d) it plays a dual role as both reporter and therapeutic gene and allows for imaging of the functional expression of the introduced gene in the most hassle free non-invasive approach possible.^{68–70} Specifically in case of neuroblastoma, branched poly-cations rooted in OEI-grafted polypropylamin dendrimers (G2-HD-OEI) coupled with the human *NIS* gene under the control of the constitutive CMV promoter has shown high transfection efficiency in an *in vitro* murine neuroblastoma model-Neuro2A.⁶⁸ The increased efficiency was coupled with a fivefold increase in iodine uptake of the cells with minimal cytotoxicity. Results also revealed high accumulation of iodine following tail-vein injection in about 85% of Neuro2A tumors.⁶⁸ It was also reported that no accumulation was observed in non-target organs like lung, liver, kidneys and so on. After two cycles of *NIS* polyplex application followed by iodine therapy, increased tumor specific iodine accumulation was observed which resulted in delayed tumor growth and improved survival of mice. Thus while the technique remains at its infancy, the scope of this cytoreductive gene therapy is broadened by the evidence of decreased tumor growth even in the case of metastatic terminal cases of neuroblastoma.⁶⁸ In addition, post delivery of *NIS* gene there was evidence of decreased blood vessel density in the tumor thus showcasing reduced angiogenesis and decrease in tumor growth stimulatory factors. This dual efficiency of the targeted radiolabeled iodine technique potentially makes it a therapeutically unique and

promising treatment option for not just neuroblastoma but all metastatic cancers.

COMBINATION OF GENE AND CELL THERAPY TO TARGET NEUROBLASTOMA

MSCs are multipotent adult stem cells of mesodermal lineage, which can be isolated from several tissues like the bone marrow, dermis, umbilical cord, peripheral blood and adipose tissue.^{71,72} These cells are capable of differentiating into fat, cartilage, bone, connective tissues and muscles throughout the body⁷³ and when delivered i.v. are able to engraft in tumor tissues and program into tumor-associated fibroblasts.^{74,75} Hence, this method makes use of genetically engineered Ad vectors and compensates for its lack of tumor targeting capacity by injecting MSCs i.v. into the system. This is observed to be effective in cases of extra-cranial metastatic neuroblastoma especially in patients with low chance of long-term survival despite high-dose chemotherapy.⁴⁴ These Ad selectively multiply in the tumor cells following their efficient delivery by the infected MSCs. Whether implanted or directly infused, MSCs have successfully been used in cases of therapies for multiple sclerosis, acute myocardial infarction, graft versus host disease and so on with good tolerance and very low toxicity.^{76–79} Concerns regarding the enhancement of the metastasis due to the infusion of MSCs directly were eliminated by inactivating the MSCs by irradiation before infusion.⁴⁴ The irradiation was also shown not to affect the replication capabilities of the Ad. Reports suggest that the delivery vehicle persisted for 48–72 h post infusion allowing not only for the MSCs to target the metastasized cancers but also allowing Ad replication while protecting the viral particles from antibodies and innate immune responses for the given window of time.^{44,80} This form of delivery has lowered the clustering of the viral particles in non-targeted tissue sites, while reaching high concentrations in the metastasized cancers. The oncolytic cycle of the Ad vector was seen to continue in the neuroblastoma cells until tumor disappearance or more possibly until the elimination of the viral particles by the immune system.⁴⁴ One of the emerging techniques of non-viral mode of gene transfer is the use of stem cells or neural stem cells as delivery vehicles to deliver therapeutic transgenes with extreme precision to tumors.⁸⁰ Neural stem cell, stem cells and progenitor cells have displayed inherent tumor tropism, which allow for safe methods of delivery bypassing the existing toxic treatment options.^{81,82}

HUMAN STUDIES

The turn of the century witnessed the final translation of gene therapies from the bench to the bedside with a number of clinical trials at both phase 1 and phase 2 showing significant progress in extending the quality time of life available to neuroblastoma patients (Table 3). In 2010, a 6-year-old boy with stage 4 neuroblastoma had an ongoing treatment of three different chemotherapy regimens including an autologous stem cell transplant. He was injected with 10^{11} viral particles of Adv513 COXLD24 (ultrasound guided) directly to tumor site.⁴⁵ The viral particles had a chimeric capsid for enhanced gene delivery to cells, the COX2 promoter driving the E1A gene and 24 deletions. Some of the side effects of the treatment included fever, diarrhea, stomach pain and grade 2 liver damage. However, 71% of mass reduction in CT imaging of primary tumor was observed with simultaneous decrement of serological tumor marker. Viral particles were observed along with elevation of cytotoxic T lymphocytes in blood 3 weeks after the therapy was administered. The patient remained alive 14 months after the therapy.⁴⁵ In another study four children with refractory metastatic neuroblastoma and resistant to three chemotherapy lines were administered with autologous MSCs carrying an oncolytic Ad ICOVIR-5,⁴⁴ which was previously demonstrated for its capacity to kill brain

Table 3. Significant gene therapy clinical trials for neuroblastoma

Clinical trial	Area	Objectives/outcome	Phase and type	Clinical trial ID
Combination therapy followed peripheral stem cell transplant in treating young patients with high-risk medulloblastoma	Mayo Clinic	To study the clinical advantages of using combination therapy of drug cocktails along with peripheral stem cell transplant to ensure higher EFS	Phase III	NCT00336024
Biological therapy and gene therapy in treating children with recurrent or refractory neuroblastoma	Fred Hutchinson Cancer Research Center	Phase-I trial to study the effectiveness of a combination of biological therapy and gene therapy techniques in neuroblastoma patients	Phase 1, 2000–2005	NCT00006480
Gene therapy in treating children with refractory or recurrent neuroblastoma	St. Jude Children's Research Hospital	Phase-I trial to monitor effectiveness in recurrent neuroblastoma patients on treatment with IL-2-modified cells	Phase 1, 1999–2007	NCT00002748
Using gene modified neuroblastoma cells for the treatment of relapsed/refractory neuroblastoma	Baylor College of Medicine	The purpose of this study is to allow for the action of lymphotactin attracting the immune cell for cell lysis of tumor cells, which can be reintroduced into the patient's body	Phase 1, 2003–2012	NCT00062855
Gene modified allogeneic neuroblastoma cells for treatment of relapsed/refractory neuroblastoma	St. Jude Children's Research Hospital	This study will utilize the concept of using the immune system to treat tumors which have a prolonged expression of tumor antigens in their malignant state. The study also explores the possibility of generating immune T cells using tumor specific protein or peptide(s) there from eradicate the tumor	Phase 1, 2005–2008	NCT00186862
Conditionally mutated HSV-1-G207		Anti-tumor activity and long-term presence of viral DNA, no toxic or adverse effect	Phase-I clinical trial	NCT00028158
BEACON—Neuroblastoma Trial	Cancer Research UK	Ongoing trial	Phase II, Interventional	NCT02308527

Abbreviation: EFS, event free survival.

tumors efficiently in preclinical models with a good safety and efficacy profile.⁸³ ICOVIR-5 exploits aberrant E2F expression in cancer cells and tight regulation of E2F in normal cells, allowing enhanced tumor selectivity while exerting a potent anti-tumor effect *in vitro* and *in vivo*.⁸⁴ E2F1 is active and regulates the expression of several target genes in high-risk neuroblastoma cases.⁴⁴ In this study, all the four children tolerated the treatment very well and one of them was in complete remission 3 years post treatment with minimal systemic toxicity.⁴⁴ Some of the other significant gene therapy clinical trials are summarized in Table 3.

CONCLUSION AND FUTURE PROSPECTS

Neuroblastoma, a solid tumor caused by rapid division of undifferentiated neuroblasts, is the most common childhood malignancy affecting children < 5 years. Several approaches and strategies developed and tested to cure neuroblastoma have met with limited success due to different reasons. Many genes are known to be involved in the onset and development of neuroblastoma and thus offer an opportunity to circumvent this disease if the expression of these genes is restored to normalcy. Gene therapy is a powerful tool for modulating the expression levels of target genes in specific cells and the same can be used to control neuroblastoma. Both viral and non-viral vector-based gene therapies have been developed and adopted to deliver the target genes into neuroblastoma cells with many of them showing promising results. These attempts have provided some hope in considering gene therapy as a new regime of treatment against neuroblastoma. While the future of neuroblastoma remains unclear the fact that quite a few gene-therapy-based treatment strategies have been validated in clinical trials yielding positive results shows that this technology could be established as an effective tool to address neuroblastoma. Concerted efforts to identify more potent candidate genes and means of delivery would make gene therapy a choice treatment with precise effects. For example, the role of hypoxia (HIF-1α) and angiogenesis (VEGFα) is well-documented in the pathogenesis of brain cancer which contributes to the disappointing clinical outcomes.^{34,85} Increased angiogenesis is critical for tumor growth and colonization in the brain, whereas hypoxia promotes a more malignant phenotype of cancer cells.^{85,86} HIF-1α is one of the most important driving forces for upregulation of VEGFα, which leads to cancer dissemination and therapeutic resistance.⁸⁷ Both VEGFα and HIF-1α could thus serve as crucial gene therapy targets that could potentially reduce tumor growth. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) could be another potential therapeutic target. It is known to play a crucial role in driving the malignant phenotype in patients with brain tumors and more importantly to positively regulate the expression of both HIF-1α and VEGFα in various tumors.^{88,89} Thus local inhibition of NF-κB could help prevent/reduce tumor spread in part by downregulating both hypoxia and angiogenesis and also reduce production of inflammatory cytokines, which can lead to tumor formation.⁹⁰ The success of such treatments will highly depend not only on timely diagnosis but also on careful selection of the appropriate gene therapy vector. For example, one of the problems with viral vectors is the host immune response, which can severely compromise the safety and efficacy of the treatment.³⁰ AAV is known to be least immunogenic with an excellent safety profile as compared with the other viral vectors. Different AAV serotypes have already been shown to efficiently infect solid human tumor tissues obtained from biopsy samples⁹¹ thus presenting itself as a good vector candidate for gene therapy in neuroblastoma. However, much research is required to optimize these vectors and make them suitable for brain tumor targeting more so when injected systemically. With a host of viral and non-viral vectors available at our disposal, it is time that we look at

gene therapy as an alternate and efficient treatment option for neuroblastoma with minimal side effects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

- Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* 2013; **13**: 714–726.
- Naldini L. Gene therapy returns to centre stage. *Nature* 2015; **526**: 351–360.
- Verma IM, Somia N. Gene therapy—promises, problems and prospects. *Nature* 1997; **389**: 239–242.
- Amer MH. Gene therapy for cancer: present status and future perspective. *Mol Cell Ther* 2014; **2**: 27.
- Louis CU, Shohet JM. Neuroblastoma: molecular pathogenesis and therapy. *Annu Rev Med* 2015; **66**: 49–63.
- Shimada H, Ambros IM, Dehner LP, Hata J, Joshi VV, Roald B *et al*. The International Neuroblastoma Pathology Classification (the Shimada system). *Cancer* 1999; **86**: 364–372.
- Fredlund E, Ringner M, Maris JM, Pahlman S. High Myc pathway activity and low stage of neuronal differentiation associate with poor outcome in neuroblastoma. *Proc Natl Acad Sci USA* 2008; **105**: 14094–14099.
- Kress TR, Sabo A, Amati B. MYC: connecting selective transcriptional control to global RNA production. *Nat Rev Cancer* 2015; **15**: 593–607.
- Solly SK, Trajcevski S, Frisen C, Holzer GW, Nelson E, Clerc B *et al*. Replicative retroviral vectors for cancer gene therapy. *Cancer Gene Ther* 2003; **10**: 30–39.
- Wold WS, Toth K. Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. *Curr Gene Ther* 2013; **13**: 421–433.
- Cross D, Burmester JK. Gene therapy for cancer treatment: past, present and future. *Clin Med Res* 2006; **4**: 218–227.
- Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P *et al*. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 2003; **302**: 415–419.
- Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nussbaum P *et al*. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 2000; **288**: 669–672.
- Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A *et al*. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 2002; **296**: 2410–2413.
- Liechtenstein T, Perez-Janices N, Escors D. Lentiviral vectors for cancer immunotherapy and clinical applications. *Cancers (Basel)* 2013; **5**: 815–837.
- Li W, Green WR. Immunotherapy of murine retrovirus-induced acquired immunodeficiency by CD4 T regulatory cell depletion and PD-1 blockade. *J Virol* 2011; **85**: 13342–13353.
- Listopad JJ, Kammertoens T, Anders K, Silkenstedt B, Willmsky G, Schmidt K *et al*. Fas expression by tumor stroma is required for cancer eradication. *Proc Natl Acad Sci USA* 2013; **110**: 2276–2281.
- Grupp SA, Prak EL, Boyer J, McDonald KR, Shusterman S, Thompson E *et al*. Adoptive transfer of autologous T cells improves T-cell repertoire diversity and long-term B-cell function in pediatric patients with neuroblastoma. *Clin Cancer Res* 2012; **18**: 6732–6741.
- Cho HS, Song JY, Park CY, Lyu CJ, Kim BS, Kim KY. Retroviral-mediated IL-2 gene transfer into murine neuroblastoma. *Yonsei Med J* 2000; **41**: 76–81.
- Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol* 2012; **12**: 180–190.
- Toes RE, Ossendorp F, Offringa R, Melief CJ. CD4 T cells and their role in antitumor immune responses. *J Exp Med* 1999; **189**: 753–756.
- Zarour HM, Ferrone S. Cancer immunotherapy: Progress and challenges in the clinical setting. *Eur J Immunol* 2011; **41**: 1510–1515.
- Yamamoto S, Suzuki S, Hoshino A, Akimoto M, Shimada T. Herpes simplex virus thymidine kinase/ganciclovir-mediated killing of tumor cell induces tumor-specific cytotoxic T cells in mice. *Cancer Gene Ther* 1997; **4**: 91–96.
- Fischer U, Steffens S, Frank S, Rainov NG, Schulze-Osthoff K, Kramm CM. Mechanisms of thymidine kinase/ganciclovir and cytosine deaminase/5-fluorocytosine suicide gene therapy-induced cell death in glioma cells. *Oncogene* 2005; **24**: 1231–1243.
- Steffens S, Sandquist A, Frank S, Fischer U, Lex C, Rainov NG *et al*. A neuroblastoma-selective suicide gene therapy approach using the tyrosine hydroxylase promoter. *Pediatr Res* 2004; **56**: 268–277.
- Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. *Annu Rev Biochem* 2012; **81**: 145–166.
- Jiang R, Xue S, Jin Z. Stable knockdown of MYCN by lentivirus-based RNAi inhibits human neuroblastoma cells growth in vitro and in vivo. *Biochem Biophys Res Commun* 2011; **410**: 364–370.
- Bell P, Moscioni AD, McCarter RJ, Wu D, Gao G, Hoang A *et al*. Analysis of tumors arising in male B6C3F1 mice with and without AAV vector delivery to liver. *Mol Ther* 2006; **14**: 34–44.
- Schlehofer JR. The tumor suppressive properties of adeno-associated viruses. *Mutat Res* 1994; **305**: 303–313.
- Sen D. Improving clinical efficacy of adeno associated vectors by rational capsid bioengineering. *J Biomed Sci* 2014; **21**: 103.
- Daya S, Berns KI. Gene therapy using adeno-associated virus vectors. *Clin Microbiol Rev* 2008; **21**: 583–593.
- Sen D, Balakrishnan B, Gabriel N, Agrawal P, Roshini V, Samuel R *et al*. Improved adeno-associated virus (AAV) serotype 1 and 5 vectors for gene therapy. *Sci Rep* 2013; **3**: 1832.
- Hendriksen EM, Span PN, Schuurin J, Peters JP, Sweep FC, van der Kogel AJ *et al*. Angiogenesis, hypoxia and VEGF expression during tumour growth in a human xenograft tumour model. *Microvasc Res* 2009; **77**: 96–103.
- Fischer I, Gagner JP, Law M, Newcomb EW, Zagzag D. Angiogenesis in gliomas: biology and molecular pathophysiology. *Brain Pathol* 2005; **15**: 297–310.
- Huang DY, Lin GJ, Huang SH, Liu LW, Ju DT, Chen YW *et al*. Inhibition of Nodal suppresses angiogenesis and growth of human gliomas. *J Neurooncol* 2011; **104**: 21–31.
- Dev IK, Dornsife RE, Hopper TM, Onori JA, Miller CG, Harrington LE *et al*. Antitumour efficacy of VEGFR2 tyrosine kinase inhibitor correlates with expression of VEGF and its receptor VEGFR2 in tumour models. *Br J Cancer* 2004; **91**: 1391–1398.
- Streck CJ, Zhou J, Ng CY, Zhang Y, Nathwani AC, Davidoff AM. Longterm recombinant adeno-associated, virus-mediated, liver-generated expression of an angiogenesis inhibitor improves survival in mice with disseminated neuroblastoma. *J Am Coll Surg* 2004; **199**: 78–86.
- Albini A, Marchisone C, Del Grosso F, Benelli R, Masiello L, Tacchetti C *et al*. Inhibition of angiogenesis and vascular tumor growth by interferon-producing cells: a gene therapy approach. *Am J Pathol* 2000; **156**: 1381–1393.
- Taylor KL, Leaman DW, Grane R, Mechti N, Borden EC, Lindner DJ. Identification of interferon-beta-stimulated genes that inhibit angiogenesis in vitro. *J Interferon Cytokine Res* 2008; **28**: 733–740.
- Streck CJ, Dickson PV, Ng CY, Zhou J, Gray JT, Nathwani AC *et al*. Adeno-associated virus vector-mediated systemic delivery of IFN-beta combined with low-dose cyclophosphamide affects tumor regression in murine neuroblastoma models. *Clin Cancer Res* 2005; **11**: 6020–6029.
- Zeng Y, Jiang J, Huebener N, Wenkel J, Gaedicke G, Xiang R *et al*. Fractalkine gene therapy for neuroblastoma is more effective in combination with targeted IL-2. *Cancer Lett* 2005; **228**: 187–193.
- Wong HH, Lemoine NR, Wang Y. Oncolytic viruses for cancer therapy: overcoming the obstacles. *Viruses* 2010; **2**: 78–106.
- Bowman L, Grossmann M, Rill D, Brown M, Zhong WY, Alexander B *et al*. IL-2 adenovector-transduced autologous tumor cells induce antitumor immune responses in patients with neuroblastoma. *Blood* 1998; **92**: 1941–1949.
- Garcia-Castro J, Alemany R, Cascallo M, Martinez-Quintanilla J, Arriero Mdel M, Lassaletta A *et al*. Treatment of metastatic neuroblastoma with systemic oncolytic virotherapy delivered by autologous mesenchymal stem cells: an exploratory study. *Cancer Gene Ther* 2010; **17**: 476–483.
- Pesonen S, Helin H, Nokisalmi P, Escutenaire S, Ribacka C, Sarkioja M *et al*. Oncolytic adenovirus treatment of a patient with refractory neuroblastoma. *Acta Oncol* 2010; **49**: 117–119.
- D'Alessio G. New and cryptic biological messages from RNases. *Trends Cell Biol* 1993; **3**: 106–109.
- Van Maerken T, Sarkar D, Speleman F, Dent P, Weiss WA, Fisher PB. Adenovirus-mediated hPNPase(old-35) gene transfer as a therapeutic strategy for neuroblastoma. *J Cell Physiol* 2009; **219**: 707–715.
- Meck MM, Wierdl M, Wagner LM, Burger RA, Guichard SM, Krull EJ *et al*. A virus-directed enzyme prodrug therapy approach to purging neuroblastoma cells from hematopoietic cells using adenovirus encoding rabbit carboxylesterase and CPT-11. *Cancer Res* 2001; **61**: 5083–5089.
- Yu D, Jin C, Leja J, Majdalani N, Nilsson B, Eriksson F *et al*. Adenovirus with hexon Tat-protein transduction domain modification exhibits increased therapeutic effect in experimental neuroblastoma and neuroendocrine tumors. *J Virol* 2011; **85**: 13114–13123.
- Zhao RY, Liang D, Li G, Larrimore CW, Mirkin BL. Anti-cancer effect of HIV-1 viral protein R on doxorubicin resistant neuroblastoma. *PLoS One* 2010; **5**: e11466.

- 51 Cook ML, Bastone VB, Stevens JG. Evidence that neurons harbor latent herpes simplex virus. *Infect Immun* 1974; **9**: 946–951.
- 52 McLennan JL, Darby G. Herpes simplex virus latency: the cellular location of virus in dorsal root ganglia and the fate of the infected cell following virus activation. *J Gen Virol* 1980; **51**: 233–243.
- 53 Parker JN, Gillespie GY, Love CE, Randall S, Whitley RJ, Markert JM. Engineered herpes simplex virus expressing IL-12 in the treatment of experimental murine brain tumors. *Proc Natl Acad Sci USA* 2000; **97**: 2208–2213.
- 54 Parikh NS, Currier MA, Mahller YY, Adams LC, Di Pasquale B, Collins MH et al. Oncolytic herpes simplex virus mutants are more efficacious than wild-type adenovirus Type 5 for the treatment of high-risk neuroblastomas in preclinical models. *Pediatr Blood Cancer* 2005; **44**: 469–478.
- 55 Sawiris GP, Sydiskis RJ, Bashirelahi N. Hormonal modulation of herpes simplex virus replication in a mouse neuroblastoma cell line. *J Clin Lab Anal* 1994; **8**: 135–139.
- 56 Qiu Z, Harms JS, Zhu J, Splitter GA. Bovine herpesvirus tegument protein VP22 enhances thymidine kinase/ganciclovir suicide gene therapy for neuroblastomas compared to herpes simplex virus VP22. *J Virol* 2004; **78**: 4224–4233.
- 57 Markert JM, Medlock MD, Rabkin SD, Gillespie GY, Todo T, Hunter WD et al. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther* 2000; **7**: 867–874.
- 58 Farrell CJ, Zaupa C, Barnard Z, Maley J, Martuza RL, Rabkin SD et al. Combination immunotherapy for tumors via sequential intratumoral injections of oncolytic herpes simplex virus 1 and immature dendritic cells. *Clin Cancer Res* 2008; **14**: 7711–7716.
- 59 Gillory LA, Megison ML, Stewart JE, Mroczek-Musulman E, Nabers HC, Waters AM et al. Preclinical evaluation of engineered oncolytic herpes simplex virus for the treatment of neuroblastoma. *PLoS One* 2013; **8**: e77753.
- 60 Koyuncu OO, Hogue IB, Enquist LW. Virus infections in the nervous system. *Cell Host Microbe* 2013; **13**: 379–393.
- 61 Toyoda H, Yin J, Mueller S, Wimmer E, Cello J. Oncolytic treatment and cure of neuroblastoma by a novel attenuated poliovirus in a novel poliovirus-susceptible animal model. *Cancer Res* 2007; **67**: 2857–2864.
- 62 Nomura M, Shimbo T, Miyamoto Y, Fukuzawa M, Kaneda Y. 13-Cis retinoic acid can enhance the antitumor activity of non-replicating Sendai virus particle against neuroblastoma. *Cancer Sci* 2013; **104**: 238–244.
- 63 Fernandez M, Porosnicu M, Markovic D, Barber GN. Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease. *J Virol* 2002; **76**: 895–904.
- 64 Zhang SC, Cai WS, Zhang Y, Jiang KL, Zhang KR, Wang WL. Engineered measles virus Edmonston strain used as a novel oncolytic viral system against human neuroblastoma through a CD46 and nectin 4-independent pathway. *Cancer Lett* 2012; **325**: 227–237.
- 65 Reichard KW, Lorence RM, Katubig BB, Peebles ME, Reyes HM. Retinoic acid enhances killing of neuroblastoma cells by Newcastle disease virus. *J Pediatr Surg* 1993; **28**: 1221–1225 discussion 1225–1226.
- 66 Tietze N, Pelisek J, Philipp A, Roedel W, Merdan T, Tarcha P et al. Induction of apoptosis in murine neuroblastoma by systemic delivery of transferrin-shielded siRNA polyplexes for downregulation of Ran. *Oligonucleotides* 2008; **18**: 161–174.
- 67 Shen M, Gong F, Pang P, Zhu K, Meng X, Wu C et al. An MRI-visible non-viral vector for targeted Bcl-2 siRNA delivery to neuroblastoma. *Int J Nanomedicine* 2012; **7**: 3319–3332.
- 68 Klutz K, Russ V, Willhauck MJ, Wunderlich N, Zach C, Gildehaus FJ et al. Targeted radioiodine therapy of neuroblastoma tumors following systemic nonviral delivery of the sodium iodide symporter gene. *Clin Cancer Res* 2009; **15**: 6079–6086.
- 69 Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003; **3**: 203–216.
- 70 Ogris M, Wagner E. Targeting tumors with non-viral gene delivery systems. *Drug Discov Today* 2002; **7**: 479–485.
- 71 Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997; **276**: 71–74.
- 72 Kuroda Y, Kitada M, Wakao S, Dezawa M. Bone marrow mesenchymal cells: how do they contribute to tissue repair and are they really stem cells? *Arch Immunol Ther Exp (Warsz)* 2011; **59**: 369–378.
- 73 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143–147.
- 74 Kidd S, Spaeth E, Dembinski JL, Dietrich M, Watson K, Klopp A et al. Direct evidence of mesenchymal stem cell tropism for tumor and wounding microenvironments using in vivo bioluminescent imaging. *Stem Cells* 2009; **27**: 2614–2623.
- 75 Bhattacharya SD, Mi Z, Talbot LJ, Guo H, Kuo PC. Human mesenchymal stem cell and epithelial hepatic carcinoma cell lines in admixture: concurrent stimulation of cancer-associated fibroblasts and epithelial-to-mesenchymal transition markers. *Surgery* 2012; **152**: 449–454.
- 76 Elnakish MT, Hassan F, Dakhallah D, Marsh CB, Alhaider IA, Khan M. Mesenchymal stem cells for cardiac regeneration: translation to bedside reality. *Stem Cells Int* 2012; **2012**: 646038.
- 77 Fazel S, Chen L, Weisel RD, Angoulvant D, Seneviratne C, Fazel A et al. Cell transplantation preserves cardiac function after infarction by infarct stabilization: augmentation by stem cell factor. *J Thorac Cardiovasc Surg* 2005; **130**: 1310.
- 78 Dulamea A. Mesenchymal stem cells in multiple sclerosis—translation to clinical trials. *J Med Life* 2005; **8**: 24–27.
- 79 Kim N, Im KI, Lim JY, Jeon EJ, Nam YS, Kim EJ et al. Mesenchymal stem cells for the treatment and prevention of graft-versus-host disease: experiments and practice. *Ann Hematol* 2013; **92**: 1295–1308.
- 80 Aboody KS, Najbauer J, Danks MK. Stem and progenitor cell-mediated tumor selective gene therapy. *Gene Ther* 2008; **15**: 739–752.
- 81 Aboody KS, Brown A, Rainov NG, Bower KA, Liu S, Yang W et al. Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci USA* 2000; **97**: 12846–12851.
- 82 Aboody KS, Najbauer J, Schmidt NO, Yang W, Wu JK, Zhuge Y et al. Targeting of melanoma brain metastases using engineered neural stem/progenitor cells. *Neuro Oncol* 2006; **8**: 119–126.
- 83 Cascallo M, Alonso MM, Rojas JJ, Perez-Gimenez A, Fueyo J, Alemany R. Systemic toxicity-efficacy profile of ICOVIR-5, a potent and selective oncolytic adenovirus based on the pRB pathway. *Mol Ther* 2007; **15**: 1607–1615.
- 84 Alonso MM, Cascallo M, Gomez-Manzano C, Jiang H, Bekele BN, Perez-Gimenez A et al. ICOVIR-5 shows E2F1 addiction and potent antiangioma effect in vivo. *Cancer Res* 2007; **67**: 8255–8263.
- 85 Hsieh CH, Shyu WC, Chiang CY, Kuo JW, Shen WC, Liu RS. NADPH oxidase subunit 4-mediated reactive oxygen species contribute to cycling hypoxia-promoted tumor progression in glioblastoma multiforme. *PLoS One* 2011; **6**: e23945.
- 86 Folkert RD. Descriptive analysis and quantification of angiogenesis in human brain tumors. *J Neurooncol* 2000; **50**: 165–172.
- 87 Jensen RL. Brain tumor hypoxia: tumorigenesis, angiogenesis, imaging, pseudo-progression, and as a therapeutic target. *J Neurooncol* 2009; **92**: 317–335.
- 88 Bhat KP, Balasubramanian V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F et al. Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer Cell* 2013; **24**: 331–346.
- 89 Xie TX, Xia Z, Zhang N, Gong W, Huang S. Constitutive NF-kappaB activity regulates the expression of VEGF and IL-8 and tumor angiogenesis of human glioblastoma. *Oncol Rep* 2010; **23**: 725–732.
- 90 Conti A, Ageunou M, La Torre D, Cardali S, Angileri FF, Buemi C et al. Expression of the tumor necrosis factor receptor-associated factors 1 and 2 and regulation of the nuclear factor-kappaB antiapoptotic activity in human gliomas. *J Neurosurg* 2005; **103**: 873–881.
- 91 Thorsen F, Afione S, Huszthy PC, Tysnes BB, Svendsen A, Bjerkvig R et al. Adeno-associated virus (AAV) serotypes 2, 4 and 5 display similar transduction profiles and penetrate solid tumor tissue in models of human glioma. *J Gene Med* 2006; **8**: 1131–1140.
- 92 Gelderblom HR. Structure and Classification of Viruses. In: Baron S (ed) *Medical Microbiology*. The University of Texas Medical Branch at Galveston, Galveston, TX, USA, 1996.
- 93 Brown JC, Newcomb WW, Wertz GW. Helical virus structure: the case of the rhabdovirus bullet. *Viruses* 2010; **2**: 995–1001.
- 94 Cassidy KA, Saunders U, Shimamura M. Deltagamma(1)134.5 herpes simplex viruses encoding human cytomegalovirus IRS1 or TRS1 induce interferon regulatory factor 3 phosphorylation and an interferon-stimulated gene response. *J Virol* 2012; **86**: 610–614.