



## Tumour Reviews

## Cancer gene therapy targeting cellular apoptosis machinery

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## ABSTRACT

The unraveling of cellular apoptosis machinery provides novel targets for cancer treatment, and gene therapy targeting this suicidal system has been corroborated to cause inflammation-free autonomous elimination of neoplastic cells. The apoptotic machinery can be targeted by introduction of a gene encoding an inducer, mediator or executioner of apoptotic cell death or by inhibition of anti-apoptotic gene expression. Strategies targeting cancer cells, which are achieved by selective gene delivery, specific gene expression or secretion of target proteins via genetic modification of autologous cells, dictate the outcome of apoptosis-based cancer gene therapy. Despite so far limited clinical success, gene therapy targeting the apoptotic machinery has great potential to benefit patients with threatening malignancies provided the availability of efficient and specific gene delivery and administration systems.

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## Introduction

Gene therapy is the definitive therapeutic practice involving the transferring of DNA or RNA directly to *in vivo* cells, or to isolated cells followed by reinfusion of these cells into human body.<sup>1</sup> Given that cancers are among the most severe threats to human health due to the unsatisfactory efficacy and inevitable systemic toxicity of traditional radio- and chemo-treatment, gene therapy has found its footing in treatment of neoplasia of divergent tissues.<sup>2</sup> Cancer cells could be modified with the genes of cytotoxic or tumor suppressor proteins, or with a class of suicidal genes in combination with prodrugs, all of which result in autonomous cell death.<sup>3,4</sup> Alternatively, the antitumor immune response could be elicited by genetic modification of malignant cells or immune cells to produce cytokines or tumor antigens.<sup>5,6</sup> However, considering the relatively weak immune levels in cancer patients, strategies that directly kill tumor cells are advantageous in the context of micro-environments educated by tumors.<sup>7</sup>

Whereas cytotoxic proteins like bacteria-derived toxins are capable of triggering cell death via diverse mechanisms, they frequently cause uncontrolled inflammation due to strong immunogenicity.<sup>8</sup> Thanks to our ever clearer understanding of apoptosis in recent years, oncologists are currently able to utilize this self-en-

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emy system to eliminate neoplastic cells.<sup>9–11</sup> Apoptosis is programmed cell death characterized by a series of morphological and biochemical changes which is attributed to accurately regulated molecular events or signaling cascades.<sup>9</sup> In fact, doctors had achieved apoptosis of tumor cells via traditional treatment much earlier than they were aware of that.<sup>11</sup> While both chemo- and radio-therapy caused massive apoptosis of tumor cells, they also undistinguishably kill normal cells.<sup>11,12</sup> Therefore, the establishment of targeted pro-apoptotic therapeutic protocols or the development of apoptosis-inducing drugs that target the tumor without causing severe impairment of the normal organism has been under way since the 1990s, and has provided novel approaches to the successful treatment of cancers.<sup>13,14</sup>

## Canonical apoptotic machinery in mammalian cells

Cells undergoing apoptosis exhibit hallmarks of morphological abnormalities, e.g. shrunken and bubbled cytoplasm, condensed nucleus, fragmented chromatin but intact membrane or organelle at the early stage.<sup>15</sup> Apoptosis is triggered by extracellular or intracellular stimuli, and thereafter the intracellular signaling, which ultimately leads to the degradation of functional proteins, collapse of cytoskeletons and fragmentation of DNA.<sup>16</sup>

## Death receptor-mediated extrinsic signal pathway

Death receptors are a class of transmembrane receptors belonging to the tumor-necrosis factor receptor (TNFR) superfamily. These receptors bind to ligands of the homotrimeric TNF protein family, e.g. Fas ligand (FasL or CD95L).<sup>17</sup> The association of FasL with

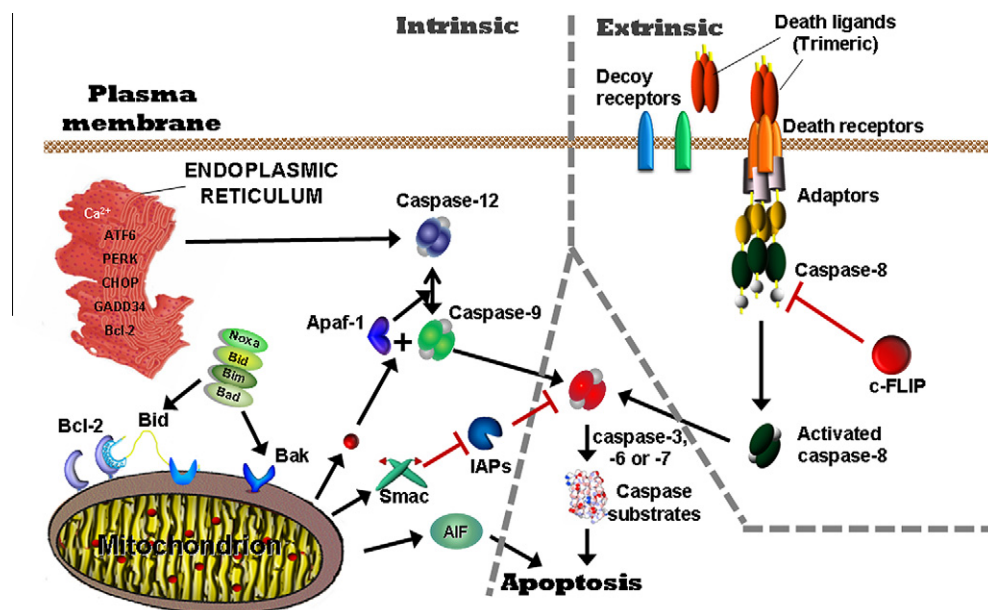
the death receptor Fas induces the trimerization of Fas, which subsequently recruits the adaptor molecule, Fas-associated death domain (FADD), via interaction between their death domains (DD). FADDs bind and activate FADD-like interleukin-1 $\beta$ -converting enzyme (FLICE), also known as caspase-8, via their death effector domains (DED), followed by a cascade of cysteine aspartate protease (caspase) activation and ultimately the cleavage of protein substrates.<sup>17–19</sup> Caspases are conserved executioners of apoptosis, existing as zymogens in mammalian cells. Upon activation, caspases recognize specific protein substrates and cleave them at certain amino acid motif after an aspartate residue. Caspases are classified into two types based on their roles in apoptotic signaling: initiator (apical) caspases like caspases-2, -8, -9, -10 and -12, and effector caspases including caspases-3, -6 and -7.<sup>19,20</sup> Initiator caspases like the aforementioned caspase-8 are activated by upstream apoptotic signaling to initiate a cascade of caspase activation. They could process and activate effector caspases, which in turn cleave divergent protein substrates involved in cell structure maintenance, metabolism and physiological functions (Fig. 1).<sup>19,21</sup>

In addition to the well-documented FasL/Fas pathway, the roles of other death receptors in apoptotic signaling have also been established.<sup>18,22,23</sup> In response to TNF engagement, TNFR1 interacts with various death domain-containing proteins to form a complex consisting of the adaptor, TNF receptor 1 associated via death domain (TRADD). While the complex preferably bind I-kappaB-kinase (IKK) to activate nuclear factor-kappaB (NF- $\kappa$ B) and promote cell survival, TRADD could also recruit other adaptors like FADD and RAIDD, which consequently trigger apoptosis via activation of caspase-8 and caspase-2, respectively.<sup>22,23</sup> The TNF-related apoptosis-inducing ligand (TRAIL) could bind 5 death receptors: TRAIL-R1 to TRAIL-R5. While TRAIL-R1 and TRAIL-R2 contain the death domain and are capable of inducing apoptosis via the FADD/caspase-8 pathway, the other three receptors, TRAIL-R3 to TRAIL-R5 serve as “decoy receptors” since they are deficient in downstream signaling and actually suppress apoptosis by competitively binding to TRAIL (Fig. 1).<sup>24,25</sup>

### Mitochondrion- and endoplasmic reticulum-related intrinsic signal pathways

The apoptotic signaling can also be initiated from inside the cells in response to stress conditions, e.g. hypoxia or survival factor deprivation, oncogene activation and DNA damage caused by radiation or chemicals. These intrinsic apoptotic pathways and the aforementioned extrinsic pathways share downstream signaling events of apoptosis execution like caspase activation. However, they are distinguished from the death receptor signaling in that cells sensor the apoptosis stimuli and activate caspases via different mechanisms (Fig. 1).<sup>15,26,27</sup>

The mitochondrion is crucially involved in the intrinsic apoptotic signaling pathways. The permeabilization of mitochondrial outer membrane is regulated by proteins of Bcl-2 family, which share one or more Bcl-2 homology (BH) domains and mediate heterodimeric interactions among different members. The Bcl-2 protein family is further divided as anti-apoptotic and pro-apoptotic protein subfamilies. The anti-apoptotic proteins like Bcl-2 and Bcl-X<sub>L</sub> are located on the surface of the mitochondrion and impede the activation and homo-oligomerization of the pro-apoptotic Bcl-2 family members, whereas most pro-apoptotic family members, such as Bax, Bad and Bid, are found in the cytosol and relocate to the mitochondrial membrane in response to apoptotic stimuli.<sup>28,29</sup> The interaction between anti-apoptotic proteins and consequently the excessive pro-apoptotic proteins result in the formation of pores on the mitochondria and the release of cytochrome C (Cyt C) from the intermembrane space. In a multi-protein platform named apoptosome, the association of cytosol Cyt C with the adaptor protein, apoptotic peptidase activating factor 1 (Apaf-1), recruits and activate caspase 9, which sequentially causes the processing and activation of effector caspases, the degradation of caspase substrates, and ultimately the collapse of cells.<sup>29,30</sup> In addition, the mitochondrion is involved in a caspase-independent apoptotic pathway governed by apoptosis-inducing factor (AIF). AIF is a protein normally located in the intermembrane space of



**Fig. 1.** The extrinsic and intrinsic pathways of apoptotic signaling. In the extrinsic pathway, extracellular death ligands bind and trimerize membrane receptors, recruit initiator caspases (caspase-8) via adaptors, and activate caspase-8 through intermolecular autoprocessing of caspases in proximity. In the intrinsic pathway, cells in stress undergo compartmental changes involving the mitochondrion and endoplasmic reticulum permeability controlled by the Bcl-2 family, which cause the activation of initiator caspases, either caspase-9 in an apoptosome complex upon release of cytochrome C, or caspase-12 by disturbed calcium homeostasis prior to a series of molecular interactions. In both extrinsic and intrinsic pathways, activated initiator caspases process and activate effector caspases, which subsequently cleave divergent protein substrates and cause apoptotic cell death.

the mitochondria, and upstream signaling causes the release of AIF to the cytosol and further to the nuclei, where it causes the destruction of chromosomal DNA (Fig. 1).<sup>31</sup>

Other organelles also play crucial roles in the self-rising apoptotic pathways. Of note is the endoplasmic reticulum (ER), which is highly sensitive to stresses that perturb cellular energy levels, the redox state or Ca<sup>2+</sup> concentration. Transient ER stress induces unfolded protein responses (UPR) and promote cell survival, while prolonged ER stress causes apoptosis via mechanisms yet to be fully defined.<sup>32,33</sup> Nevertheless, the participants of this unique apoptotic pathway involve the pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) in the initiation phase, the transcription factor C/EBP homologous protein (CHOP), growth arrest and DNA damage-inducible gene 34 (GADD34), tribbles-related protein 3 (TRB3) and Bcl-2 family members in the commitment phase, and finally activated caspases for the execution of apoptosis (Fig. 1).<sup>32,33</sup>

### Regulators of apoptotic signaling

The apoptosis machinery is precisely regulated by protein members of varied families. Like the anti-apoptotic members of Bcl-2 families, the inhibitor of apoptosis (IAP) proteins represent another family negatively regulating apoptosis. IAPs could normally bind and prevent activation of caspases (Fig. 1).<sup>34</sup> In particular, the X-linked inhibitor of apoptosis (XIAP) binds caspase-9, -3 and -7, thereby inhibiting their activation and suppressing apoptosis.<sup>35</sup> Survivin, another member of IAPs, could bind and block the activation of caspases-3 and -7.<sup>36</sup> The second mitochondria-derived activator of caspase (SMAC)/Diablo and the high temperature requirement protein-A2 (HTRA2) counteract the effect of IAPs.<sup>37</sup> The FLICE-inhibitory protein (FLIP) with alternate names Casper/I-FLICE/FLAME-1/CASH/CLARP/MRI, binds to FADD and caspase-8, and thereby inhibits its death receptor-mediated apoptosis. As a target gene of NF- $\kappa$ B, FLIP also dictates the outcome of TNF signaling, i.e. whether cells continue to survive or undergo apoptosis.<sup>38</sup>

The generalized apoptosis regulators also comprise particular oncoproteins and tumor suppressors.<sup>39,40</sup> While oncoproteins inhibit apoptosis by elevating signals of cell growth and proliferation, tumor suppressors could promote apoptosis by attenuating growth signals or directly acting on the apoptotic machinery. Among its wide roles in genome stability and cell behaviors, p53 serves as a sensor of cellular stress and a critical activator of the intrinsic apoptotic pathway.<sup>39</sup> p53 could be phosphorylated and stabilized by DNA checkpoint proteins in response to DNA damage, and initiate the gene transcription of pro-apoptotic Bcl-2 subfamily, e.g. Bax and Bid, and other tumor suppressors like phosphatase and tensin homolog (PTEN). PTEN overexpression leads to apoptosis via negatively regulating PI3 kinase pathway or by associating with Bax in the mitochondrion.<sup>40</sup>

### Deregulated apoptosis in carcinogenesis

Both the ontogenesis of multicellular organisms and maintenance of normal morphology and function of organs require concurrent cell proliferation and apoptosis, which are balanced by requisite regulatory mechanism. During carcinogenesis, however, these mechanisms were disturbed and cells undergo uncontrolled proliferation while exhibiting resistance to apoptosis. The insufficiency of apoptosis is attributed to either lack of pro-apoptotic stimuli in the *in vivo* environment or the blockade of cellular apoptotic pathways.<sup>13,41,42</sup>

Neoplastic cells have evolved diverse mechanisms to alter apoptotic signaling via the extrinsic or intrinsic pathway.<sup>13,41,42</sup> The downregulation of Fas or Fas ligand is found in numerous

malignancies, which could be attributed to traditional genetic mechanisms involving polymorphism or to epigenetic modifications.<sup>43</sup> In osteosarcoma cells, the downregulation of Fas by microRNA-20a (miR-20a) allow migrating cells to survive and form metastases in FasL-positive lung microenvironment.<sup>44</sup> The expression of FasL/Fas has thus been implicated in prognosis evaluation of patients with varied cancers.<sup>45</sup> Meanwhile, the expression of FasL may also be upregulated in cancers, which contributes to excessive apoptosis of T cells and thus serves as a mechanism of immune escape.<sup>46</sup> The adaptor protein, FADD, also plays multifaceted roles in carcinogenesis. FADD downregulation was found in various cancers like renal cell carcinoma.<sup>47</sup> However, it is intriguing that the upregulation, phosphorylation and nuclear localization of FADD are also associated with carcinogenesis and poor outcome of patients, probably reflecting a role of FADD in cell cycle regulation.<sup>48</sup> The downregulation of another adaptor, Apaf-1, was also found to correlate with the progression of certain clinical malignancies like breast adenocarcinomas.<sup>49</sup> As executioners of apoptosis, caspases are frequently deficient in cancer cells. For example, caspase-8 is absent in a class of small cell lung carcinomas, which is predictive of resistance to pro-apoptotic treatment.<sup>50</sup> Somatic caspase-8 mutations have been well-documented in lung, stomach, breast and pancreatic cancers. Also reported in cancer tissues are the mutations in the coding or regulatory regions of caspases-3, -7 and -9, which hamper transcription of the genes, disrupt translation of full-length protein or interfere with the activation of the resulting mutants.<sup>51</sup>

Bcl-2 family members and regulators of the apoptotic machinery have been found aberrantly expressed or frequently mutated in carcinoma cells. While overexpression of Bcl-2 or Bcl-xL is associated with the development or metastasis of cancers, the absence or inactivation of the pro-apoptotic Bcl-2 family members, such as Bax, Bid and Bim is involved in carcinogenesis of varied tissues.<sup>52,53</sup> Similarly, overexpression and activation of the apoptosis inhibitors, like XIAP and c-FLIP, are found in neoplastic cells and have proved a prognostic significance in leukemia and other malignancies.<sup>54,55</sup>

### Strategies targeting the apoptotic machinery in carcinoma cells

The deficient apoptotic signaling and thereby the inadequate apoptosis in cancer cells provide the rationale for gene therapy to target the apoptotic machinery. In theory, a majority of the molecules involved in apoptotic signaling or regulation can be targeted. However, since the apoptotic pathways and regulatory mechanisms are conserved in mammalian cells and common in different types of human cells of an individual, strategies that constrain the above molecular targeting to cancer cells are preferably needed.<sup>56–58</sup> These cell targeting strategies are so crucial that they can even dictate the outcome of a therapeutic protocol, reminiscent of the cytotoxicity and limited success of the prevalent molecule-targeting anticancer drugs, e.g. the small molecule kinase inhibitors.<sup>56,59</sup>

#### *Molecular targeting: the spear or the shield*

Given that deficiencies in the apoptosis machinery underlie the occurrence of numerous carcinomas, it is rational to correct or compensate for these genetic deficiencies in cancer therapy. Nevertheless, the targeting to cellular apoptotic signaling could also be valid in the treatment of cancers with relatively normal apoptosis machinery, considering that this machinery could be utilized for enforced “suicide” of cancer cells.<sup>56,57</sup>

The induction of cancer apoptosis is among the most common approaches in cancer gene therapy or immunotherapy. The gene encoding an inducer, mediator or executioner of apoptosis is

routinely introduced in cancer cells to overcome the deficiency of its endogenous counterpart.<sup>60–62</sup> While theoretically a majority of the ligand of death receptors can initiate apoptosis, TNF-related apoptosis-inducing ligand (TRAIL, also designated CD253) is the most frequently used apoptosis inducer in cancer treatment.<sup>63</sup> Because TRAIL-R1 and TRAIL-R2 are mainly expressed in transformed cells and the decoy receptors TRAIL-R3, TRAIL-R4 and TRAIL-R5 on normal cells, TRAIL has been found to kill a wide variety of tumor cells with minimal effects on adjacent normal cells.<sup>24,63</sup> Apoptosis executioner genes, like caspases, are cytotoxic to cancer cells regardless of the status of the apoptosis machinery, i.e. whether there is an inherent defect in the classical apoptotic pathway.<sup>20,64</sup> In addition, a proapoptotic gene expression may reverse the malignant phenotype caused by persistent growth signaling, provided the accumulating data supporting the crosstalk between signals responsible for cell growth and apoptosis resistance.<sup>65–67</sup> In particular, the epidermal growth factor receptor (EGFR) was found to upregulate c-FLIP and result in TRAIL resistance via promoting the expression of tissue transglutaminase (TGM2) in lung cancers.<sup>67</sup> Conversely, targeting the apoptotic machinery will also contribute to growth and proliferation inhibition in addition to apoptosis induction in cancer cells.<sup>56,60</sup>

While an initiative attack of carcinomas by an apoptotic gene provides a spear against the deficient apoptotic machinery in cancer cells, the functional inactivation of the endogenous apoptosis inhibitors may serve as a shield to prevent the adverse effect of these molecular abnormalities.<sup>68–71</sup> Provided the frequent correlation of carcinogenesis with high levels of these negative regulators of apoptosis, small inhibitory RNAs like the antisense, siRNAs or microRNAs can be developed to target these regulators including the apoptosis inhibitors and Bcl-2 family antiapoptotic members.<sup>68</sup> For instance, siRNA-mediated silencing of FLIP<sub>L</sub> (long form) in neuroblastoma cells promotes apoptosis and restored the sensitivity to other apoptosis inducers like TRAIL and FasL.<sup>69</sup> The gene of a dominant negative mutant represents another loss of function strategy for these apoptosis inhibitors. Of note is the gene of dominant negative survivin, which induced apoptosis, sensitized cells to proapoptotic regents or radiotherapy, and served as a effective antigen derivation of dendritic cells to elicit antitumor immunity.<sup>70</sup> Although not yet applied to apoptosis inhibitors, genes encoding intracellular antibodies will prove useful in suppression of these inhibitors in the treatment of related cancers.<sup>71</sup>

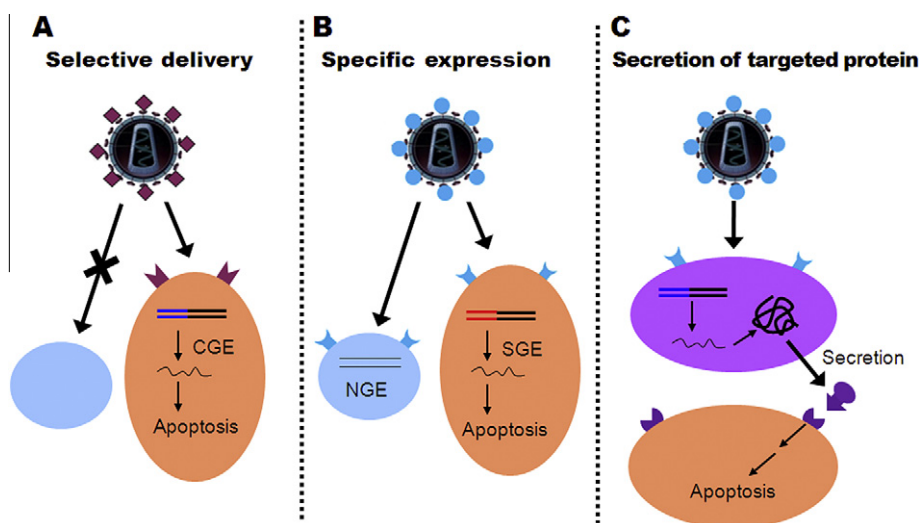
### Cellular targeting: the devil but not the normal

Since most neoplastic cells share common apoptotic machinery with normal cells, strategies that target the molecular intervention to carcinoma cells are required. The targeting of cancer cells in gene therapy could be achieved in the levels of either gene delivery or expression. Alternatively, cancer cells can be targeted by a chimeric cytotoxic protein secreted by genetically modified autologous cells.<sup>72–74</sup>

#### Specific delivery of apoptotic genes in carcinoma cells

The successful delivery of a therapeutic gene is the premise of in vivo cancer gene therapy. Both viral particles and non-viral carriers can be modified to specifically recognize cancer cells.<sup>75,76</sup> The viral particles bind host cells via their envelope proteins, which could be modified to target carcinoma cells or tumor vascular epithelium. The cell tropism of the wild-type viruses could be modified by replacing the natural receptor-binding sequence of the envelope glycoprotein with the peptide recognizing the neovascular cells, e.g. somatostatin and RGD motif, or with ligands/scFvs that binds tumor-specific receptors or antigens (Fig. 2A).<sup>77,78</sup> The viral envelopes can also be modified by charged or surface-modifying polymers like polyethylene glycol (PEG), followed by conjugation with a tumor-targeting peptide. In terms of this, a hybrid adeno-associated virus phage vector (AAVP) was developed to target tumor vasculature through the modified envelope containing a av integrin ligand RGD-4C motif. This vector was then used for delivery of the tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) gene, thus reducing the systemic toxicity of TNF $\alpha$ .<sup>79</sup>

Non-viral gene carriers, especially the prevalent nanoparticles consisting of lipid-like materials, are alternate systems for targeted delivery of apoptotic genes.<sup>80–84</sup> Using the recombinant protein asialoglycoprotein (Asor), which targets asialoglycoprotein receptor (ASGPR) present only on the surface of hepatocytes, Peng et al.<sup>80</sup> generated a target vehicle, and demonstrated that it could deliver the apoptin gene specifically into hepatocellular carcinoma cells, but not normal hepatocytes or malignant cells of other origins. Fay et al.<sup>81</sup> coated the Colloidal nanoparticle with a DR5 antibody, which was used for delivery of cytotoxic drugs, and found this recombinant carrier could directly trigger apoptosis of colorectal cancer cells. Goldberg et al.<sup>82</sup> used lipidoids to deliver



**Fig. 2.** Gene therapy strategies targeting cancer cells. (A) Viral or non-viral carriers were modified to delivery the apoptotic gene selectively into cancer cells but not normal cells. (B) Genes were delivered to both cancer and normal cells, but the regulatory elements allow the expression of the apoptotic gene specifically in cancer cells. (C) Autologous cells were modified to secrete chimeric protein, which targets cancer cells and induces apoptotic cell death. CGE, constitutive gene expression; NGE, no gene expression; SGE, specific gene expression. Blue lines, constitutive gene regulatory elements; red lines, regulatory elements enforcing gene expression specifically in cancer cells.



siRNAs targeting the Poly (ADP-ribose) polymerase (PARP)-1, which is a caspase substrate and plays apoptosis-preventing roles by facilitating repair of damaged DNA. In delivery of hTRAIL gene, Han et al.<sup>83</sup> generated a tumor-targeting carrier, PAMAM-PEG-T7, consisting of a T7 peptide recognizing the transferrin receptor specifically expressed on tumor cells. The T7 peptide was conjugated to polyethylene glycol-modified polyamidoamine dendrimer. Lu et al.<sup>84</sup> described a cationic albumin-conjugated pegylated nanoparticles (CBSA-NP), which could deliver the expression cassette of a given gene like TRAIL across the blood–brain barrier in an orthotopic glioma model.

#### *Selective expression of apoptosis-triggering genes in carcinoma cells*

The efficient expression of a therapeutic gene in target cells is another pivotal procedure for cancer gene therapy. By replacing the constitutive promoter with cell type-specific regulatory elements, the expression of a gene selectively in cancer cells can be achieved.<sup>73</sup> A tissue-specific promoter or enhancer is useful to avoiding undesired expression of the cytotoxic gene in organs or tissues irrelevant to the primary tumors (Fig. 2B). By placing adenoviral E1a, E4 and E1b genes under the control of two individual prostate-specific enhancing sequences, Li et al.<sup>85</sup> generated prostate-restricted replicative adenovirus (PRRA). This recombinant adenovirus was then used to deliver FasL, which is controlled by the same regulatory elements as the above virus proteins, thereby achieving the expression specificity in prostate cells in addition to the targeted gene delivery.

The regulatory element of an oncogene provides a more reliable way to express apoptotic gene in cancer cells. In targeted gene therapy of bladder and prostate cancers, the promoter of Cox-2, a gene overexpressed in numerous cancer cells, was used to control the expression of inducible forms of caspases, like caspases-3 and -9, thereby circumventing the mutation of upstream signals causing TNF- $\alpha$  resistance.<sup>86</sup> Using a glial cell-specific GFAP promoter combined with Herpes Simplex Virus type 1 (HSV-1)-based amplicon vector in which the activation of the transgene expression is regulated by a G0/G1-specific transcriptional repressor protein termed cell cycle-dependent factor 1, CDF-1, Ho et al.<sup>87</sup> achieved both glioma-specific and cell cycle-dependent expression of the pro-apoptotic molecules, FasL and FADD.

Unfortunately, small RNAs targeting the apoptosis inhibitory system normally require the promoters recognized by RNA polymerase III (like U6 or H1 promoter) rather than the routine RNA pol II promoters.<sup>88</sup> While shRNAs driven by a tumor-specific RNA pol II promoter have recently been developed by replacing the CMV promoter of commercialized shRNA expressing vectors with the survivin or stathmin promoter, their efficiency to drive shRNA expression in vivo is still open to further investigation.<sup>89</sup> Nevertheless, RNA pol II promoter-based small RNA-expressing systems by embedding the RNA-coding sequence in that of a microRNA have been developed.<sup>90</sup> Alternatively, the shRNA coding sequence could be inserted in the 3' untranslated region (UTR) of a therapeutic gene, and coexpressed from a single mRNA to inhibit tumor cell proliferation or growth.<sup>91</sup>

#### *Secretion of tumor-targeted proapoptotic proteins by modified autologous cells*

While routine gene therapy introduces a gene encoding a cellular protein, it is not uncommon that a therapeutic gene is delivered to produce a protein which could be secreted and function in the extracellular compartment or by re-entering the cells.<sup>61,62</sup> In terms of cancer gene therapy, a chimeric pro-apoptotic protein can be

generated by fusing a cancer-targeting moiety with an apoptotic-inducing molecule. Cells, either normal cells or cancer cells themselves, can then be genetically modified to secrete the above protein (Fig. 2C). This strategy is of particular value when a systemic treatment is required, for instance, in cases with wide distant metastasis.<sup>92–100</sup>

The chimeric genes encoding a class of secreting proapoptotic proteins have been generated so far by utilizing the tumor-targeting characteristics of antibodies or peptides. Li et al.<sup>92</sup> described a lentivirus-mediated modification of cells to secrete a chimeric antibody targeting the death receptor DR5. The light and heavy chains of the recombinant antibody were linked by a 2A/furin self-processing peptide, thereby facilitating its expression from one single vector and the assembly after cleavage by furin. Tumor-targeted TRAIL can also be generated by fusing an  $\alpha_v$  integrin ligand peptide RGD-L to the amino terminal of TRAIL.<sup>93</sup> Shah et al.<sup>94</sup> constructed recombinant TRAILS, which retain in the endoplasmic reticulum and remain inactive unless processed by a viral protease, achieving inducible release of TRAIL and controlled pro-apoptotic activity and the bystander tumoricidal effects. In contiguous studies focusing on gene therapy of erbB2/HER2-overexpressing cancers, Yang's group<sup>74,95–100</sup> generated a series of proapoptotic chimeric proteins by fusing a single chain antibody against HER2 and active apoptotic proteins spaced by a translocation domain from natural protein toxin or a specific sequence which could be recognized and processed by furin, a proprotein convertase involved in endosome–cytosol translocation of endocytosed proteins. In principle, these chimeric proteins selectively bind HER2-overexpressing breast cancer cells and internalize via endocytosis, which is followed by cytosol translocation after proteolytic processing by furin. The released active apoptotic proteins like active caspases and truncated Bid culminate in triggering apoptosis of cancer cells. These chimeric proteins are advantageous over immunotoxins in that they kill tumor cells in an intrinsic physiologic manner, resulting in relatively weak immunogenicity and minor systemic toxicity over repeated treatments. A fusion gene encoding a signaling peptide-flanked version of the chimeric proteins was then used to modify in vivo cells, resulting in production of the protein and induced apoptosis of carcinoma cells.<sup>74,95–100</sup>

The secreting proapoptotic protein strategy could be extended to a further theoretical significance when special types of cells are modified to secrete the pro-apoptotic proteins. Modification of T lymphocytes with these apoptosis-triggering genes resulted in the generation of a class of tumor killer cells with characteristics of both cellular immunity and humoral immunity, given that these cells could secrete the proapoptotic protein and exert a systemic tumoricidal effect after homing to the peripheral lymphoid tissues.<sup>100,101</sup> Groth et al.<sup>102</sup> generated TRAIL-secreting lymphocytes, which was linked by a bispecific antibody, EpCAM $\times$ CD3, to tumor cells expressing EpCAM, leading to apoptosis of tumor cells. In addition, the CD3-recognizing moiety of the bispecific antibody also stimulates the proliferation of these modified lymphocytes, thus expanding the antitumor effect of lymphocytes. The mesenchymal stem cells (MSCs) were modified by two independent groups to secrete TRAIL in the treatment of pancreatic cancers and gliomas, respectively.<sup>103,104</sup> Mohr et al.<sup>103</sup> generated TRAIL-secreting MSCs, which could infiltrate both tumor and lymphatic tissues to target primary tumors as well as disseminated cancer cells in a pancreatic cancer model, and the pro-apoptotic capacity was further improved by combination with siRNA-mediated silencing of XIAP. Grisendi et al.<sup>105</sup> used modified adipose-derived mesenchymal stromal/stem cells (AD-MSC) as a cellular vector of TRAIL to overcome the short lifespan of recombinant TRAIL protein in treatment of cervical and pancreatic cancers. In an early study by Ehtesham et al.,<sup>106</sup> neural stem cells were modified to produce TRAIL and were reinfused to treat gliomas, which was proved

efficient in eradicating both primary tumors and tumor satellites via induction of apoptosis.

### Clinical trials and perspectives

By virtue of the progress in laboratory studies on cancer gene therapy targeting the apoptotic machinery, clinicians now have the opportunities to evaluate the therapeutic potential of a growing list of pro-apoptotic gene reagents on cancer patients.<sup>7,61,62</sup> Due to a confound role of TNF- $\alpha$  in inflammation and cancer development, it remains a debate whether TNF- $\alpha$  potentially benefits cancer patients, which has been even complicated by the recent discovery that TNF- $\alpha$  is critically involved in vascular permeability regulation. In contrast to the limited success of recombinant TNF- $\alpha$  protein due to high toxicity, adenovirus-delivered TNF- $\alpha$ , designated TNFerade, has showed emphatic therapeutic potentials in advanced, metastatic or recurrent solid tumors including pancreatic cancer, esophageal cancer, soft tissue sarcoma and melanoma.<sup>107–112</sup> A phase III clinical trial combined with chemoradiation (CRT) in locally advanced pancreatic cancer indicated an encouraging trend of overall survival in favor of the TNF-treated group compared with CRT alone (Table 1).<sup>107</sup> The antitumor capac-

ity of the well-documented apoptosis inducer, TRAIL, was also assessed in phase I/II clinical trials both as a recombinant human protein or as a therapeutic gene delivered by adenovirus. However, it is frustrating that the sponsors, Genentech and Amgen, terminated their studies on rhTRAIL after a phase I clinical trials on advanced cancers. While a phase I study of adenovirus-delivered TRAIL is still under way for treatment of organ confined prostate cancer following radical prostatectomy, development of fusion gene encoding TRAIL conjugated to a tumor-targeting moiety, or strategies for selective delivery to the neoplastic tissues will definitely add weight to the opportunity of clinical success.<sup>63,113</sup> A constitutively active form of Bcl-2-interacting killer (Bik), which is a proapoptotic Bcl-2 subfamily member, was demonstrated to synergized with lapatinib in elimination of breast cancer initiating cells, and has already been involved in a phase I study deciphering its therapeutic effect on advanced pancreatic cancer (Table 1).<sup>114</sup>

In parallel with the above gene augmentation strategies, the inactivation of an anti-apoptotic gene was also addressed clinically. A locked nucleic acid (LNA)-based or phosphorothioate-modified antisense molecules against Bcl-2 was approved for clinical studies in a variety of cancers including relapsed or refractory lymphocytic leukemia, and have shown promising response

**Table 1**  
Clinical cancer gene therapy trials targeting the apoptotic machineries.

Gene	Trial ID	Trial essentials	References
TNF- $\alpha$	US-0010	Genetically modified autologous cancer cells, phase I, closed	Rosenberg <sup>125</sup>
	US-0399	Adenovirus-delivered, intratumoral, combined with radiotherapy, locally advanced, recurrent, or metastatic solid tumors, phase I, closed	Sharma et al. <sup>109</sup> , Senzer et al. <sup>111</sup> , Hecht et al. <sup>112</sup>
	US-0457	Adenovirus-delivered, intratumoral, combined with radiotherapy, adjunct to surgery, soft tissue sarcoma, phase I, closed	Mundt et al. <sup>108</sup>
	US-0549	Adenovirus-delivered, intratumoral, combined with chemotherapy, prior to esophagectomy for locally advanced esophageal cancer, phase II, open	<a href="http://www.abedia.com/wiley/record_detail.php?ID=1179">http://www.abedia.com/wiley/record_detail.php?ID=1179</a> ; data not released
	US-0730	Adenovirus-delivered, intratumoral, combined with radiotherapy, metastatic melanoma, phase II, open	MacGill et al. <sup>126</sup> Clinical data to be released
	US-0750	Adenovirus-delivered, intratumoral, combined with radio- and chemo-therapy, unresectable recurrent head and neck cancer, phase I/II, open	<a href="http://www.abedia.com/wiley/record_detail.php?ID=1380">http://www.abedia.com/wiley/record_detail.php?ID=1380</a> ; data not released
	NCT00051480	Adenovirus-delivered, intratumoral, combined with radio- and chemo-therapy, esophageal cancer, phase II, completed	<a href="http://clinicaltrials.gov/ct2/show/NCT00051480">http://clinicaltrials.gov/ct2/show/NCT00051480</a> ; data not released
NCT00051467	Adenovirus-delivered, intratumoral, combined with radio- and chemo-therapy, pancreatic cancer, phase III, completed	<a href="http://clinicaltrials.gov/ct2/show/NCT00051467">http://clinicaltrials.gov/ct2/show/NCT00051467</a> ; data not released	
TRAIL	US-0603	Adenovirus-delivered, intratumoral, clinically organ confined prostate cancer undergoing radical prostatectomy, phase I, open	<a href="http://www.abedia.com/wiley/record_detail.php?ID=1233">http://www.abedia.com/wiley/record_detail.php?ID=1233</a> ; data to be released
Caspase-9	US-0849	T cells modified with a caspase-FKBP fusion gene (iCasp9), depletion of T cells upon GVHD occurring in relapsed acute leukemia patients receiving haploidentical stem cell transplantation, phase I, open	Di Stasi et al. <sup>124</sup>
	NCT00710892	Allodepleted T cells transduced with inducible caspase 9 suicide gene, acute lymphoblastic leukemia and non-Hodgkin's lymphoma, phase I, open	Di Stasi et al. <sup>124</sup>
	NCT01494103	Donor T cells with caspase-9 suicide gene (DOTT1), leukemia, phase I	<a href="http://clinicaltrials.gov/ct2/show/NCT01494103">http://clinicaltrials.gov/ct2/show/NCT01494103</a> ; data not released
Bik	NCT00968604	Lipofection-delivered, intravenous, advanced pancreatic cancer, phase I, open	<a href="http://clinicaltrials.gov/ct2/show/NCT00968604">http://clinicaltrials.gov/ct2/show/NCT00968604</a> ; data not released
Bcl-2 XIAP	UK-0130	Naked LNA antisense, relapsed or refractory chronic lymphocytic leukemia, phase I/II, open	O'Brien et al. <sup>116</sup> , Moreira et al. <sup>68</sup>
	UK-0111	Antisense RNA (AEG35156/GEM640), intravenous, advanced tumors, phase I, open	Dean et al. <sup>120</sup>
	UK-0174	AEG35156, combined with chemotherapy, advanced pancreatic cancer, phase I/II, open	Tamm <sup>123</sup>
	NCT00882869	AEG35156, combined with sorafenib, advanced hepatocellular carcinoma, phase I/II, completed	<a href="http://clinicaltrials.gov/ct2/show/NCT00882869">http://clinicaltrials.gov/ct2/show/NCT00882869</a> ; data not released
	NCT00558545	AEG35156, combined with paclitaxel, advanced breast cancer, phase I/II, terminated	Perez et al. <sup>127</sup>
	NCT00557596	AEG35156, combined with gemcitabine, advanced pancreatic cancer, phase I/II, terminated	<a href="http://clinicaltrials.gov/ct2/show/NCT00557596">http://clinicaltrials.gov/ct2/show/NCT00557596</a> ; data not released
	NCT00372736	AEG35156, combined with docetaxel, locally advanced, metastatic, or recurrent solid tumors, phase I, completed	<a href="http://clinicaltrials.gov/ct2/show/NCT00372736">http://clinicaltrials.gov/ct2/show/NCT00372736</a> ; data not released
	NCT00768339	AEG35156, relapsed or refractory chronic lymphocytic leukemia and indolent B-cell lymphomas, phase I/II, terminated due to slow recruitment	<a href="http://clinicaltrials.gov/ct2/show/NCT00768339">http://clinicaltrials.gov/ct2/show/NCT00768339</a> ; data not released
	NCT00385775	AEG35156, advanced cancers, phase I, terminated due to dosing cohort exceeded current dosing in other trials.	<a href="http://clinicaltrials.gov/ct2/show/NCT00385775">http://clinicaltrials.gov/ct2/show/NCT00385775</a> ; data not released
	NCT01018069	AEG35156, combined with cytarabine and idarubicin, AML following failure of a single standard dose cytarabine based frontline induction regimen, phase II, terminated due to failed to reach endpoints	Schimmer et al. <sup>121,128</sup>

rates with good tolerability.<sup>115–118</sup> Also in a phase I/II clinical studies are the XIAP antisense, namely AEG35156, in treatment of advanced cancers like pancreatic cancers, and the data concerning patients' responses remain to be released (Table 1).<sup>119–123</sup>

Modification of cells with apoptotic genes before infusion to human bodies, i.e. cell therapy, also annotated the role of controlled apoptosis in clinical cancer gene therapy. In a phase I clinical trial to treat relapsed acute leukemia, Di Stasi et al. reconstituted patients' immune system with genetically modified donor T cells mixed with haploidentical stem-cells. These T cells express a fusion protein of caspase-9 and FK-binding protein, which dimerizes, become activated and commit apoptotic cell death upon treatment with a small molecule drug, AP-1903. This suicidal mechanism was then utilized to eliminate the infused cells in case of adverse events, e.g. the frequently occurring graft-versus-host diseases (GVHD) in hematopoietic stem cell transplantation.<sup>124</sup>

Although apoptosis of cancer cells underlies a majority of conventional therapeutic protocols, gene therapy strategies or medications directly targeting the apoptotic apparatus are still in their infancies.<sup>3,13,61,62</sup> Despite currently the clear understanding of the apoptotic signaling has depicted explicit targets for genetic intervention, the efficacy and safety problem of gene carriers have impeded the prosperity of proapoptotic gene medicine. A future breakthrough in the development of gene delivery and expression systems, which are rigorously neoplastic cell-targeting, applicable to systemic administration and amenable in formulation to clinical demands, will bring apoptosis-based cancer gene therapy out of the embarrassing situation.<sup>75,76,93</sup> Nevertheless, while tumor cells may be removed when exposed to simply cytotoxic reagents, apoptosis induction is competitively advantageous since apoptotic cells die autonomously and rarely elicit inflammatory response or systemic toxicity.<sup>129,130</sup> Provided the characteristics of apoptotic cell death and progress in related technologies, gene therapy targeting the apoptotic machinery will gain substantial momentum in the ongoing war towards threatening cancers.

### Conflict of interest statement

The authors declared no conflict of interest.

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