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Antitumor effects of apoptin expressed by the dual cancer-specific oncolytic adenovirus – a review

J.-B. FANG[1], G.-Z. ZHU[1], Y.-L. ZHU[1], Y.-Q. LI[1], C. SHANG[2], B. BAI[1], N.-Y. JIN[1,2], X. LI[1,2]

[1]Academician Workstation of Jilin Province, Changchun University of Chinese Medicine, Changchun, Jilin, China
[2]Laboratory of Molecular Virology and Immunology, Institute of Military Veterinary Medicine, Academy of Military Medical Science, Changchun, Jilin, China

Abstract. – Apoptin is a small molecular weight protein derived from chicken anemia virus, which can induce the apoptosis of transformed cells and tumor cells and leave primary and nontransformed cells unharmed. Apoptin's cell localization depends on its own phosphorylation state and cell type. In tumor cells, phosphorylated apoptin enters the nucleus and induces apoptosis. While, in normal cells apoptin mainly exists in the cytoplasm. Apoptin, as a disordered protein in cells, interacts with many proteins in cell signal pathways to induce apoptosis of tumor cells. The specific mechanism of apoptosis induced by apoptin has not been completely elucidated. Therefore, apoptin has become a potential anticancer agent. This review summarizes the research results of apoptin in our laboratory and reveals the specific antitumor mechanism of apoptin expressed by oncolytic virus vector on a variety of tumor cells and mouse models.

Key Words:
Apoptin, Cancer, Apoptosis, Phosphorylation, Oncolytic adenovirus.

Introduction

Apoptin is a small molecular weight apoptosis inducing protein derived from chicken anemia virus (CAV). CAV belongs to a single stranded circular DNA virus. CAV was described firstly in Japan in 1974[1]. CAV genome was cloned by Mathieu H.M. Noteborn's lab in 1990[2]. CAV includes three partially overlapped genes, which encode three proteins. They are VP1, VP2, and VP3, respectively. VP1 is a 51kD coat protein. VP2 is a 24kD the dual cancer-specific phosphatase related to virus replication and a scaffold protein for assembled coat protein. VP3 is a 13kD unstructured protein that can induce apoptosis of transformed cells and tumor cells. Therefore, VP3 is also called apoptin[3]. Apoptin causes depletion of thymocytes and erythroblastoid cells in young chickens and result in severe anemia, thymus atrophy weight loss until death. It is found that carrying tumors and the intra-tumoral expression of apoptin causes tumor regression in chickens[4,5]. Noteborn et al[6] reveal that VP3 accumulates in the nucleus and VP3 alone is sufficient to induce apoptosis in chicken mononuclear cells. It was later found that VP3 can also induce apoptosis of human tumor cells but normal cells[7]. Apoptin has become a potential antitumor drug due to its specific antitumor effect. In this paper we summarize the recent work of our laboratory in the field of apoptin antitumor.

The Structure of Apoptin

Apoptin, composed of 121 amino acids with a molecular weight of 13.6kd, is rich in proline, serine and threonine, and is not homologous with any known protein in cells. There are two nuclear localization signals (NLS) at the C-terminal, including NLS1 (amino acid 82-88) and NLS2 (amino acid 111-121). There are also two nuclear export signals (NES) at both the N-terminal and C-terminal, which are necessary for promoting nuclear transport[8]. In addition, several phosphorylation sites such as threonine 108 (Thr-108) in the C-terminal can be phosphorylated by protein kinases in cells to regulate the biological activity of apoptin. There is also a short leucine rich stretch (LRs) at the N-terminal, which is

Corresponding Authors: Li Xiao, MD; e-mail: lixiao06@mails.jlu.edu.cn
Ningyi Jin, MD; email: ningyik@126.com

an important part of the interaction apoptins and between apoptin and other proteins[9]. The polymer of apoptin can promote apoptosis and is stable in tumor cells. But it is not stable or even degradation in normal cells. Polymer of apoptin also can combine with DNA to form a super structure. However, their binding sites and super structure functions also need to be further explored. The molecular structure of apoptin is shown in Figure 1[4].

Apoptosis of Tumor Cells Induced by Apoptin

The mechanism of apoptosis induced by apoptin has not been fully understood. It is generally believed to be related to subcellular localization and phosphorylation[10]. Apoptin has broad range of antitumor properties, can specifically induce apoptosis of transformed or tumor cells, and has no effect on normal cells[11]. Apoptin is mainly located in the nucleus of tumor cells, while in normal cells it is mainly in the cytoplasm. Subcellular localization is necessary, but not the only condition, because apoptin cannot induce normal cell apoptosis even if it is located in the normal nucleus under the guidance of exogenous nuclear localization signal[12]. Apoptin can induce tumor cell specific apoptosis which is independent of p53. So apoptin can kill tumor cells with loss of p53 function or cells with p53 mutation[13]. In normal cells, apoptin is mainly located in the cytoplasm. Although it enters the nucleus, it is easy to export of the nucleus. In the cytoplasm, apoptin is degradated by proteasome pathway[14]. Treated with proteasome inhibitor bortezomib showed that the degradation of apoptin decreased in normal cells, but no change in apoptin content was found before and after using proteasome inhibitor in tumor cells[15]. The phosphorylated apoptin enters the nucleus and binds with other protein partners to induce apoptosis of tumor cells. Apoptin Thr-108 phosphorylation covers the nearby NES region, which leads to the accumulation of apoptin in the nucleus, and finally leads to apoptosis of tumor cells[16]. There are two ways to phosphorylate apoptin[17]. One is to be directly phosphorylated by protein kinaseCβ (PKCβ); the other is to be phosphorylated by cyclin dependent kinase2 (CDK2) which phosphorylated through PI3K/Akt. PI3K inhibitors not only inhibit the phosphorylation of CDK2, but also inhibit the cytotoxic effect of apoptin[18]. Apoptin interacts directly with the p85 regulatory subunit (SH3) domain of PI3K through its proline rich sequence, which is essential for the phosphorylation of apoptin and its cytotoxic activity[19,20]. Akt and apoptin are transported to the nucleus, where akt activates CDK2, which in turn phosphorylates T108 of apoptin[21]. There are two mechanisms for Akt to activate CDK2: The one is directly phosphorylation of CDK2 and the other is phosphorylation-induced degradation of the inhibitor p27(Kip1) of CDK[22-24]. Numerous studies have confirmed that phosphorylation of apoptin is necessary for its induction of apoptosis[25]. Lai et a[26]l have shown that VP2 directly interacts with apoptin in the nucleus, and down regulates apoptosis by dephosphorylation status of apoptin. Therefore, some protein kinases that can phosphorylate apoptin in cells will also become new targets of anticancer agents in the future.

Apoptosis is a process of hydrolysis, regulated by caspase enzyme (caspase) which is involved in the initiation and execution of apoptosis[27,28]. In recent years, it has been found that there are two classic apoptotic pathways and an endoplasmic reticulum (ER) stress pathway. Excessive ER stress can induce apoptosis, which is a new apoptotic signal pathway. This pathway includes the mechanisms of unfolded protein response and calcium initial signal[29,30]. Two classical pathways are also the main apoptotic pathways. One of them is extrinsic pathway,

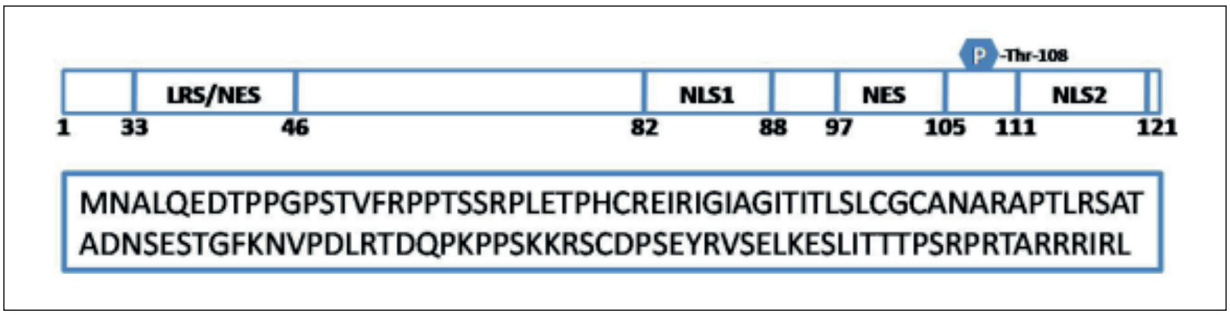

Figure 1. The primary structure of apoptin.

which starts from the death receptor. The death receptor, such as Fas, binds to its ligand FasL, and activates caspase-8 through a series of cell signals, then activates downstream caspases 3, 6, 7 to produce a cascade reaction[31]. The other is intrisic pathway, which starts from mitochondria. When cells are stimulated by internal or external apoptotic signals, the permeability of outer membrane of mitochondrion will be changed. At the same time, cytochrome c (Cyt c) of mitochondria will be released into cytoplasm[32]. The apoptosome is formed by Cyt c, the apoptosis protease activating factor (Apaf-1), and precursors of caspase 9, which activates caspase 9[33,34]. After caspase 9 activated, the downstream caspase cascade was reactivated. Both extrinsic and intrisic pathways will eventually lead to the activation of downstream caspase effectors, then apoptosis[35]. It is usually considered that apoptin induced apoptosis of tumor cells mainly through the intrisic pathway[36]. The upstream stage of apoptosis induced by apoptin is related to the interaction of other proteins in some cell signaling pathways, such as PI3K/Akt pathway. The down-regulation of Hsc70 can inhibit apoptin-induced phosphorylation and activation of Akt. But the importance of this mechanism needs further study[37,38]. In addition, apoptin also interacts with other proteins to induce apoptosis. In particular, Nur77, an orphan receptor, regulates cell growth and apoptosis. Under various apoptosis stimulation, phosphorylated Nur77 is transferred from nucleus to mitochondria, directly or indirectly activating intrinsic apoptosis pathway, promoting apoptosis rather than inhibiting apoptosis by changing the conformation of bcl-2[39,40]. Treatment of MCF-7 cells with TAT-apoptin triggers the movement of Nur77 to mitochondria[41]. Furthermore, inhibiting the expression of Nur77 can protect cells from apoptin induced killing. Although Nur77 may play a potential role in apoptin induced cell death pathway, the exact mechanism is still unclear[37,42]. In fact, apoptin entering the nucleus also interacts with other protein molecules. These protein include DEDAF (Death effector domain–associated factor), Nmi(N-Myc-interacting protein)[43], APC/C (anaphase-promoting complex/cyclosome) and PML (Promyelocytic leukemia protein)[25]. All of these explain apoptin specific killing effect of tumor cells. Except for phosphorylation, apoptin also has SUMO modification. The interaction between apoptin and Sumo may be related to the replication of CAV[44].

Antitumor Effect of Apoptin Expressed by Dual Cancer-Specific Oncolytic Adenovirus

No matter the type of tumor, apoptin seems to be able to sense the early events of gene transformation and induces specific apoptosis of cancer cells[45]. Therefore, apoptin is undoubtedly a potential anti-tumor drug, especially when chemotherapy is not sensitive. The effect of promoting tumor cell apoptosis is not dependent on the p53 tumor inhibition pathway, nor is it inhibited by the overexpression of BCL2[46]. However, apoptin cannot enter the cell by itself, so the appropriate carrier is needed for taking apoptin into the tumor cell. The carrier not only needs to have an ability to bring and express apoptin efficiently in malignant cells, but also be safe and have no toxic on normal cells. At present, there are some carriers. Such as non replicating adenovirus[47], adeno-associated virus carrier[48], non viral gene delivery system[49], and apoptin peptides[50,51]. In recent years, our Lab has done some work on the inhibition of tumor by apoptin and constructed a series of apoptin expression vectors, including non viral vector (pVVP3IL-18[52], pVVP3IL-18HN[53], pIRApotinHNIL18[54] TNG/pUAS-Apoptin[55], pVAX1-Apoptin[56], pApoptin + pIL-18[57], CAtin[58-60], and pIRES-UAS-EGFP[61],) and viral vector (Recombinant fowlpox virus (vFV-Apoptin)[62-64], Ad-Apoptin-PEG3p-E1a-[65], and conditionally replication competent adenoviruses(RCRA) also called dual cancer-specific oncolytic adenovirus(Ad-hTERT-E1a-Apoptin)[66]. A series of experiments were carried out *in vitro* and *in vivo* using these carriers. The results showed that expressed apoptin could suppress effectively primary and metastatic tumors. Furthermore Ad-hTERT-E1a-Apoptin has the characteristic of conditional replication, which overcomes the disadvantage of non replication and becomes an attractive strategy for cancer gene therapy. Herein we discuss recent findings on apoptin's molecular mechanism of action, especially the role of Ad-hTERT-E1a-Apoptin.

The structural characteristics of Ad-hTERT-E1a-Apoptin, in briefly, human telomerase reverse transcriptase promoter (hTERTp) was inserted into the upstream of human adenovirus type 5 E1a gene, apoptin was placed in the downstream of CMV promoter of RAPAD.I adenovirus shuttle plasmid. The expression boxes containing hTERTp and E1a were connected to RAPAD.I adenovirus vector system shuttle plasmid containing apoptin. The recombinant adenovi-

rus Ad-hTERT-E1a-Apoptin was obtained by co-transfection of pAd-hTERT-E1a-Apoptin and RAPAD.I adenovirus vector system genomic skeleton plasmid into HEK-293 cells. The purification and titration of the amplified virus were performed using Adeno-X Virus Purification kit (BD Bioscience Clontech) and Adeno-X Rapid titer kit (BD Bioscience Clontech), respectively. Among them, RAPAD.I is the adenovirus vector. apoptin is the effector of specific tumor suppressor gene; hTERTp is the tumor specific promoter; E1a is the essential gene for adenovirus replication. The structural diagram of recombinant adenovirus is shown in Figure 2[66].

In the dual cancer-specific oncolytic adenovirus (Ad-hTERT-E1a-Apoptin), the hTERT promoter (the 5' flanking region of the hTERT gene between positions -283 to -78) drives E1a and the cytomegalovirus (CMV) promoter to drive apoptin expression.

Telomerase is an RNA-dependent DNA polymerase that elongates 5'-TTAGGG-3' telomeric DNA [67]. hTERT is a rate-limiting and catalytic component of telomerase enzyme. Most normal human somatic cells lack telomerase activity due to the tight transcriptional suppression of the hTERT gene. However, hTERT expression and telomerase activation are observed in up to 90% of human malignances, giving them an unlimited proliferation ability[68]. Therefore, its promoter has been used for tumor specific expression of transgenes.

Adenovirus-based vectors are the most widely used platforms in gene delivery. However, non-replicating adenoviruses are seldom effective in eradicating tumor cells[69]. Therefore, it usually requires high concentration and multiple

Figure 2. Schematic diagram of organization elements in recombinant adenoviruses.

administration to produce a significant anti-tumor response. But this program usually induces an anti-virus immune response, resulting in the neutralization of the virus vector in the subsequent immunity, which is toxic to the tissues. In order to overcome these limitations, conditional replication active adenovirus has been developed and widely evaluated. These viruses replicate specifically in tumor cells, then dissolve tumors and release virus progenies, further infecting and destroying adjacent cancer cells[66].

Apoptin is the product of VP3 gene of chicken anemia virus, which has specificity and high efficiency for a variety of transformation and malignant cells. The role of apoptin is as mentioned above. Numerous studies showed that the inhibition of apoptin on many kinds of tumors by inserting into various vectors are of great significance for tumor gene therapy.

The antitumor effect of the Ad-hTERT-E1a-Apoptin was investigated *in vitro* and *in vivo* using different type cancer cells and animal models which shown in Table I.

We investigated the inhibitory effect of Ad-hTERT-E1a-Apoptin on a variety of tumors, including melanoma[66], gastric cancer[70], prostate cancer[71,74], colorectal cancer[72], lung cancer[73,77,80], breast cancer[75,78,81], cervical cancer[76], hepatoma[79], bladder cancer (to be published elsewhere). Ad-hTERT-E1a-Apoptin specifically inhibited the

Table I. Antitumor effects of Ad-hTERT-E1a-Apoptin in different cancer cell and tumor model in our lab.

Cancer types/tumor models	References
Human melanoma cells A375/B16 mouse melanoma cells/C57BL/6 mice	[66]
Human gastric cancer cells SGC7901/BALA/c nude mice	[70]
Human Prostate cancer cells PC-3/mouse cell line RM-1/C57BL/6 mice	[71]
Human CRC cells SW1116 cells/mouse CRC cell CT26/C57BL/6mice	[72]
Human lung adenocarcinoma cells A549/BALB/c nude mice	[73]
Human Prostate cancer cells PC-3 luc cells/BALB/c nude mice	[74]
Human breast cancer cells MCF-7 cells/human normal mammary epithelial cells MCF-10A	[75]
Human cervical carcinoma cells HeLa cells	[76]
Human lung adenocarcinoma cells A549 luc cells	[77]
Human breast cancer cells MCF-7 luc cells	[78]
Human hepatoma cells QGY-7703 and SMMC-7721/BALB/c nude mice	[79]
Human lung squamous carcinoma NCI-H226/BALA/c nude mice	[80]
Human breast cancer cells MCF-7 luc cells and MDA-MB-231 luc cells/BALB/c nude mice	[81]

growth of tumor cells but not normal cells. Hoechst, Annexin V, JC-1 staining, and Western blotting were employed to analyze the inhibition pathway of Ad-hTERT-E1a-Apoptin on tumor cells. Infection of tumor cells with Ad-hTERT-E-1a-Apoptin caused a marked increase in the activity levels of caspases-3, 6 and 7[66,70-81]. Significant quantities of cytochrome c were detected in the cytosol of the Ad-hTERT-E1a-Apoptin-infected cancer cells[70,72,79]. Transwell assay showed that Ad-hTERT-E1a-Apoptin inhibited tumor migration and invasion[73,80,81]. In summary, the Ad-hTERT-E1a-Apoptin can promote tumor cell apoptosis through intrisic pathway. Apoptin acts on the downstream of caspases cascade reaction. Once apoptosis start, even if the start-up process of caspases cascade reaction is inhibited, cells can still die eventually.

In addition, we also studied the antitumor effects of the combination of Ad-hTERT-E1a-Apoptin and chemotherapy drugs, including cisplatin, doxorubicin, gemcitabine, and peclitaxel[73,78,80,81]. Ad-hTERT-E1a-Apoptin can not only inhibit the growth of tumor cells and promote the apoptosis of tumor cells, but also has a significant synergistic effect on tumor inhibition with chemotherapy drugs. At the same time, it also reduces the toxicity of chemotherapy drugs[73,80,81].

Also, luc-cells were used for animal model study[74,77,78,81]. Bioluminescent imaging is a visualization technique for tracing cells and tissue, and gene behavior *in vivo*[82]. The animal model based on bioluminescence principle is an ideal model for evaluating the effect of antitumor drugs more intuitively. The tumor cells were transfected with a plasmid containing the luciferase gene, after select culture, the best luciferase activity and stability cells were obtained. The labeled tumor cells were injected into the experimental animals to establish a visualized tumor model. Animal experiments showed that whether Ad-hTERT-E-1a-Apoptin alone or Ad-hTERT-E1a-Apoptin combined with chemotherapy drugs can inhibit tumor growth, prolong the survival time of tumor bearing mice and reduce the toxicity of chemotherapy drugs.

At the same time, the pharmacology and toxicology of Ad-hTERT-E1a-Apoptin in animal models were investigated by using BALB/c mice, Wistar rats, Hartley guinea pigs, and beagles. The safety evaluation demonstrated that Ad-hTERT-E1a-Apoptin has no adverse effects on the central nervous system, or the cardiovascular, respiratory, and gastrointestinal systems. Ad-hTERT-E1a-Apoptin did not induce obvious adverse effects in the experimental animals. These results showed that Ad-hTERT-E1a-Apoptin is a safe anti-tumor therapeutic agent[83].

It is an interesting finding, recent research results in our laboratory show that Ad-hTERT-E-1a-Apoptin not only induces tumor cell death through apoptosis, but also regulates tumor cells autophagy process[75,76]. However, it depends on tumor type. In MCF-7 cells, the expression level of autophagy related proteins, including beclin-1, microtubule related protein 1a / 1b light chain 3, autophagy related 4B cysteine peptidase and autophagy related protein 5, increased significantly in the early stage (6-12h) after apoptin stimulation. Meanwhile, the expression of apoptosis related proteins decreased. With increasing the time, autophagy related proteins decreased, and apoptosis increased at the 24 hours. Therefore, apoptin may enhance autophagy and inhibit apoptosis in the early stage of MCF-7 cells. As time goes on, apoptosis is dominant. Similar results also were found in human cervical carcinoma cells (HeLa cells). However, when it comes to bladder cancer cells, including UMUC-3 and T24 cell lines, autophagy-related protein increased continuously after Ad-hTERT-E1a-Apoptin treatment with correspondingly enhanced apoptosis (to be published elsewhere).

Autophagy is one of the most important processes in eukaryotes, which is evolutionarily conserved for the turnover of intracellular materials. In this process, some damaged proteins or organelles are wrapped by the autophagic vesicles of double membrane structure, and then sent to lysosomes for degradation and recycling. However, the roles of autophagy in the regulation of survival or cell death, particularly in cancer cells, are considerably more complicated. Autophagy is usually triggered by nutritional deficiency, reactive oxygen species, hypoxia, drug stimulation and endoplasmic reticulum stress through complex signaling pathways[84]. Changes in autophagy mechanism may lead to a variety of pathological states, such as neurodegeneration, aging and cancer [85]. Autophagy usually accompanied by cell death, also known as autophagic cell death or type II programmed cell death.

The dual cancer-specific oncolytic adenovirus expressing apoptin induced cell death may involve both apoptosis and autophagy. Apoptosis and autophagy are two different forms of cell death, which are independent and interact with each other. Many genes play an important role

in the mutual transformation of two different forms[32,86,87]. The crosstalk between apoptosis and autophagy is critical to the cell fate but complicated by their contradictory roles under some conditions. Both apoptosis and autophagy as partners affect each other and influence cell homeostasis, cargo clearance of dying cells, as well as clinical therapeutics[88,89]. So far, it is not clear whether autophagy and apoptosis are directly or through a third-partner interaction. The crosstalk between apoptosis and autophagy induced by Ad-hTERT-E1a-Apoptin needs to be investigated. Figure 3 is a deduction of the relationship between autophagy and apoptosis.

In tumor cells, apoptin is phosphorylated by a protein kinase and it translocates to the nucleus where it accumulates. Subsequently apoptin activates the apoptotic machinery through the mitochondria resulting in cell death, in other hand, at the early stage, apoptin activates autophagy pathway. The crosstalk between autophagy and apoptosis induced by the Ad-hTERT-E1a-Apoptin is yet to be found. Black dots represent cytochrome c.

Conclusions

Cancer is a major public health problem worldwide and is the main leading cause of death in the world[90]. The treatment of cancers has been the focus of attention. Currently, the most common treatment for cancer is surgery combined with radiotherapy and chemotherapy. Although these methods are effective, they also have significant side effects. One of the main goals of cancer treatment is to eliminate malignant cells while minimizing the damage to normal cells. The ideal anti-tumor preparation must have high-efficiency and specific killing effect on tumor cells and no damage to normal cells of the body. With the development of molecular biology, cell biology and virology, gene therapy has become a new cancer treatment method. Among which oncolytic virus therapy shows great advantages and is expected to become a reliable cancer treatment method. Gene therapy with apoptin offers unique advantages over current approaches for cancer therapy. Apoptin, specifically killed tumor cells while leaving normal cells unharmed, has become a potential antitumor drug. However, more important is how to efficiently deliver apoptin into cancer cells. As mentioned before, a tumor specific apoptosis inducing gene (apoptin) and a cancer specific promoter (hTERT) were inserted into the adenoviral vector to obtain a novel dual specific antitumor oncolytic adenovirus, i.e. Ad-hTERT-E1a-Apoptin.

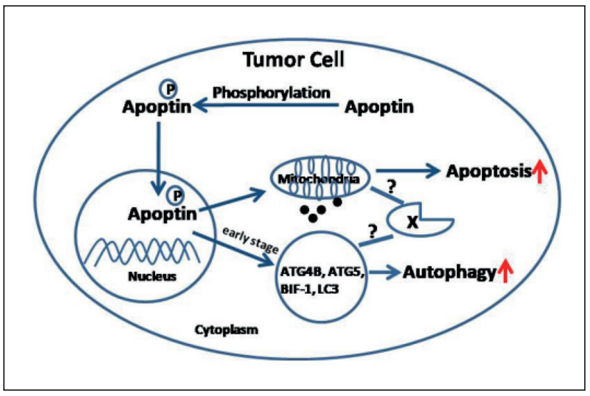

Figure 3. The model of Ad-hTERT-E1a-Apoptin activation in tumor cells.

In a series of experiments with different tumor cells and animal models, Ad-hTERT-E1a-Apoptin can obviously induce tumor cell apoptosis and prolong the survival time of tumor bearing mice. Combined with some chemotherapy drugs, Ad-hTERT-E1a-Apoptin has synergistic effect and reduces the toxicity of chemotherapy drugs. These studies show that Ad-hTERT-E1a-Apoptin is a promising tumor treatment drug, which can effectively and specifically deliver apoptin to tumor cells, overcome the non replication of adenovirus vector, so that apoptin can maintain a certain content to eliminate tumor cells.

In conclusion, Ad-hTERT-E1a-Apoptin have the following excellent characteristics: (1) As a oncolytic adenovirus, it can specifically replicate in tumor cells and kill tumor cells. (2) Due to the addition of apoptin gene, it has a targeted killing effect on tumor, and so-called dual cancer-specific oncolytic adenovirus. (3) It is safe and reliable. (4) Combined with other anti-tumor drugs or chemotherapy drugs, it has synergistic effect and reduces the toxicity of chemotherapy drugs. (5) Compared with cell immunotherapy such as CAR-T, the production and the treatment costs are low.

According to our previous experimental results, Ad-hTERT-E1a-Apoptin can stimulate tumor cell apoptosis and regulate autophagy. However, the details of the relationship between apoptosis and autophagy in the presence of apoptin need to

be further explored. To study the anti-tumor mechanism of Ad-hTERT-E1a-Apoptin is of great significance not only to understand the occurrence and development of tumor, but also to formulate new tumor treatment strategies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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