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REVIEW ARTICLE

p53 as the Focus of Gene Therapy: Past, Present and Future

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Abstract: ***Background*:** Several gene deviations can be responsible for triggering oncogenic processes. However, mutations in tumour suppressor genes are usually more associated to malignant diseases, with p53 being one of the most affected and studied element. p53 is implicated in a number of known cellular functions, including DNA damage repair, cell cycle arrest in G1/S and G2/M and apoptosis, being an interesting target for cancer treatment.

***Objective*:** Considering these facts, the development of gene therapy approaches focused on p53 expression and regulation seems to be a promising strategy for cancer therapy.

***Results*:** Several studies have shown that transfection of cancer cells with wild-type p53 expressing plasmids could directly drive cells into apoptosis and/or growth arrest, suggesting that a gene therapy approach for cancer treatment can be based on the re-establishment of the normal p53 expression levels and function. Up until now, several clinical research studies using viral and non-viral vectors delivering p53 genes, isolated or combined with other therapeutic agents, have been accomplished and there are already in the market, therapies based on the use of this gene.

***Conclusion*:** This review summarizes the different methods used to deliver and/or target the p53 as well as the main results of therapeutic effect obtained with the different strategies applied. Finally, the ongoing approaches are described, also focusing on the combinatorial therapeutics to show increased therapeutic potential of combining gene therapy vectors with chemo or radiotherapy.

Keywords: p53, apoptosis, non-viral vectors, viral vectors, gene therapy.

1. INTRODUCTION

Cancer results from the accumulation of mutations that can occur during decades and have the ability to inactivate tumour suppressor genes and activate oncogenes. Tumour suppressor genes are responsible for the inhibition of cell growth and/or induction of cellular apoptosis to prevent cancer formation. Amongst all the tumour suppressor genes, one of the most important is the p53, which acts as a sensor of DNA damage and other cellular and metabolic stresses including hypoxia, oncogenetic activation and nutrient deprivation. In response to stress, p53 can induce cell cycle arrest and subsequent DNA repair, senescence or apoptosis, depending on the damage and cellular context (Fig. **1**) [1].

Also, if the p53 protein is dysfunctional, it could result in genomic instability which is a hallmark of cancer [2]. One of the most important reasons for deep attention that is paid to p53 by the research groups dedicated to cancer investigation is the fact that 50% of the patients with cancer contain various inactivating mutations in their p53-encoding gene, while the other 50% possess defective components in post-translational modification of p53 protein or alterations in p53 signaling pathway [3]. Therefore, it is very important to re-establish the p53 expression and activity in cancerous cells.

To perform this, gene therapy has been considered as a promising therapeutic option and some practical examples were already studied and successfully applied.

Gene therapy comprises the replacement or addition of a correct copy of the abnormal gene, with the purpose of restoring the genetic information, thus reverting the associated disease. This genetic-based therapeutic approach intends to increase the quality of life and also the life expectancy of the treated individual [4, 5].

In what concerns the delivery of the therapeutic gene, it could be done directly on the patient, however, if nucleic acids are delivered in a free form, they can suffer from rapid extra and intracellular enzymatic degradation, which drastically reduces the gene available and the expression of the target protein. Moreover, due to the high molecular weight and anionic nature, gene-based products are usually difficult to achieve cellular uptake. Finally, even the small amount of gene reaching the cells can suffer degradation in the endosomal/lysosomal compartments [6]. Regarding that and, to overcome the barriers associated with the administration of

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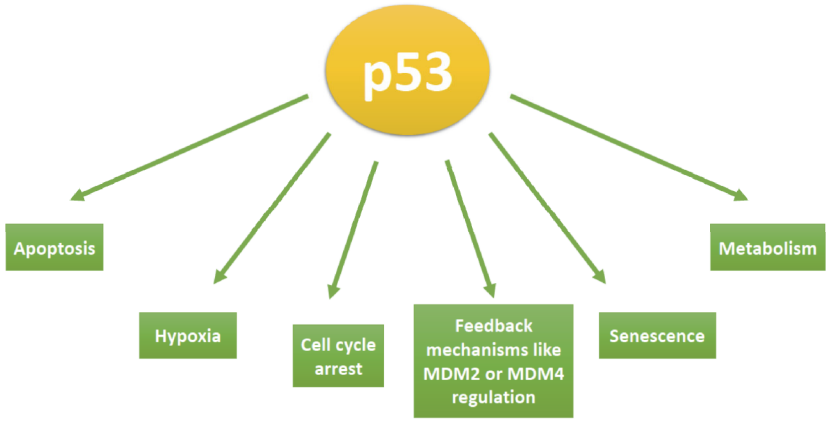

Fig. (1). Cellular processes regulated by p53.

genetic material, the main strategy under study is the association of the genetic material with suitable delivery systems. In general, these delivery systems should be characterized in terms of safety (biocompatibility and biodegradability), resistance to degradation, capability to access the appropriate cellular compartment, specificity towards the target sites and ability to selectively modulate the expression of the target gene or to express the protein for a desired period of time [6, 7].

Up until now, several clinical research studies using viral vectors for the delivery of p53 have been conducted and there are already available in the market some p53-viral vectors-based products, such as Advexin and Gendicin. However, the application of viral vectors can induce high immunogenicity and, can promote high rate of pre-existing immunity which limits their clinical use, increasing the need to create systems with the same efficiency but without these deleterious effects [8, 9]. Regarding that, non-viral vectors can represent a good alternative to the viral systems. Actually, non-viral vectors present many advantages when compared with the viral ones, mainly due to the absence of viral components, the lack of immunogenicity, the lower production costs and easier manufacturing processes.

In general, viral and non-viral strategies proved to be promising in the reestablishment of the p53 levels in cancer cells, being the viral vectors the ones that are used in more clinical studies. However, researchers around the world have started to focus on non-viral vectors and in the specific delivery to target cells.

2. P53 PROTEIN - AN INTRODUCTION TO P53 BIOLOGICAL ACTIVITY

p53 was regarded as an oncogene in the past, however, more recently, it has been proved repeatedly that p53 is, in fact, a very powerful tumour suppressor involved in apoptotic and senescent processes [10, 11]. The p53 protein is considered the "genome guardian" since when the cells are exposed to stressful stimuli, p53 is activated through post-transcriptional modifications increasing its stability and activity [12].

P53 is a protein that contains 393 amino acids and, is structurally and functionally composed of four different domains: an acidic amino-terminal domain used in the transcriptional activation, a DNA-binding domain in the central space, a tetramerization domain and finally, a C-terminal regulatory domain rich in basic amino acids which play an important role in the regulation of the core DNA-binding domain (Fig. **2** outlines the structure of p53). Regarding these domains, the N-terminus mainly interacts with transcriptional factors, like TFIID (TBP, TAFs) which is extremely important in the regulation of gene expression and working in the transcriptional machinery. Another protein that binds in this terminal is MDM2 (murine double minute 2) which is responsible for the negative regulation of the transcriptional activity of p53 [13]. The tetrameric part is, in fact, a dimer of a dimer bond to four repeats of a DNA sequence [14]. The C-terminal domain is responsible for the p53 binding to specific DNA sequences in the central domain. Particular changes in the C-terminal of this domain, like deletion or phosphorylation, have been described as being responsible for the activation of the sequence-specific DNA-binding by the central core domain [15]. The DNA-binding region is the central core of p53 and is the place where most of the p53 missense mutations were found [16]. It is made up of immunoglobin like β-sandwich of two antiparallel β-sheets, providing a scaffold for a flexible DNA-binding surface. This surface is made of two large loops stabilized by a zinc atom and a loop-sheet-helix motif. The zinc binding is extremely important for the correct folding and requires the reduction of thiol groups on cysteines [17].

p53 also plays a critical role in DNA repair and, in the control of cell cycle progression, being particularly responsible for the regulation of the cyclin-dependent kinase (CDK) p21 gene expression. The p21 binds to different CDK/cyclin complexes promoting their inhibition, and consequently, blocking the cell cycle progression [18, 19]. In addition, p53 also directly influences the transcription of CDKs, responsible for the phosphorylation of tumour suppressors of the Rb family [20]. Thus, low levels of these CDKs will not allow the activation of pRb, and consequently, induce cell cycle arrest. In G2/M checkpoint the p53 has also an important role since it interferes with the expression of several intertwined targets, including Cdc25C, 14-3-3δ and GADD45. The Cdc25C is a mitosis-promoting phosphatase that dephosphorylates and activates cyclin B1/cdc2 complex. In case of DNA damage, Cdc25C is inhibited, resulting in the G2/M arrest [21].

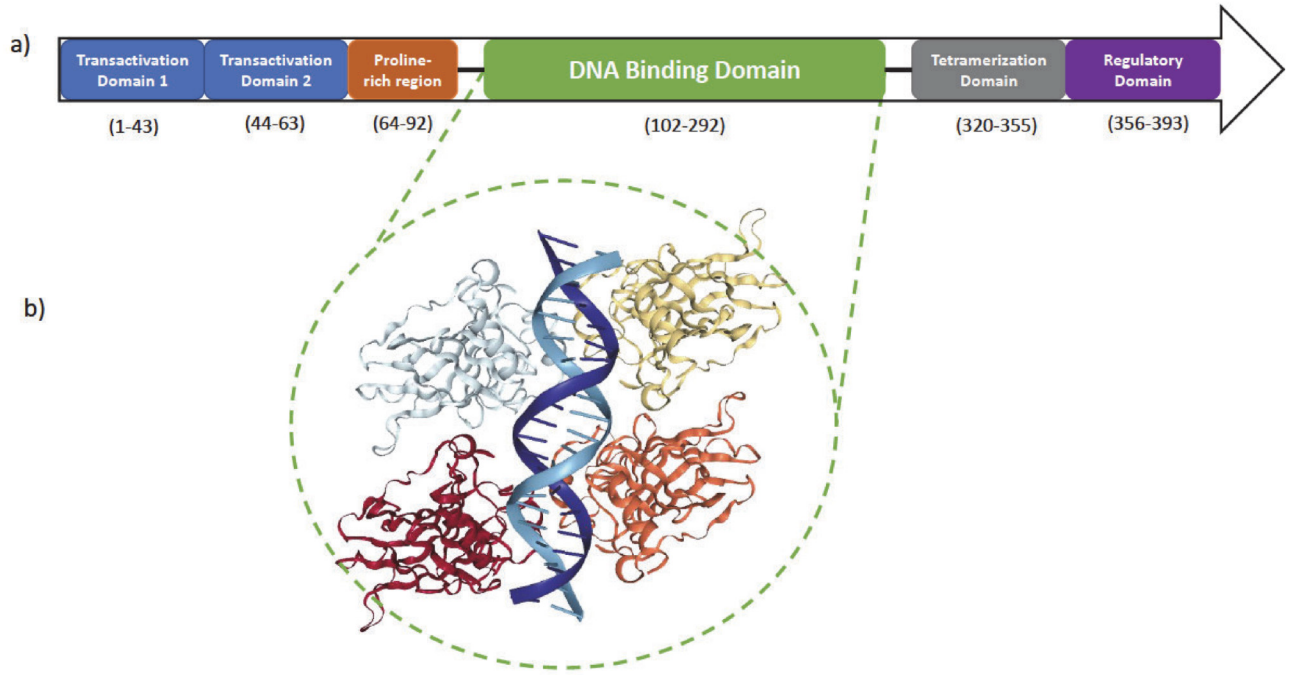

Fig. (2). p53 based structure is made of 393 amino acids organized in different domains: transactivation domain 1 and 2, a proline-rich region, DNA binding domain, tetramerization domain and also the regulatory domain. The interactions between this protein and DNA is performed through the DNA binding domain.

In the absence of stress or damage, the p53 is present in very low concentrations and is highly unstable. This is mainly due to the interaction with other proteins like the MDM2 that is one of the most important regulators of the p53 activity and stability. Usually MDM2 levels are very high in human cancers. MDM2 is an E3 ubiquitin ligase that binds to the N-terminus of p53 through a hydrophobic pocket domain, transferring mono-ubiquitin tags onto lysine residues [22]. Hence, when cells are in a non-stress environment, p53 function is kept in a basal state which is achieved due to the constant action of this MDM2-p53 loop that is responsible for the elimination of the p53 in excess [22]. The degradation of p53 is dependent on MDM2 levels, and a reduced binding of MDM2 to p53 reduces the E3 activity, leading to an inhibition of ubiquitylation function. Regarding this regulatory effect of MDM2 in p53 levels and activity, it could be important to target this interaction as a way to develop a therapeutic strategy [2].

MDMX, also known as MDM4, is a protein from MDM2 family that also binds to the N-terminal of p53 and was found to be related to the inhibition of p53 since it contributes to p53 degradation [23]. Similarly, to MDM2, MDMX is an important negative regulator of p53, and this negative regulation is one mechanism by which MDMX acts as an oncogene to transform cells when overexpressed. MDM4 has also another important role, which is the MDM2 stabilization, and is important to be referred that it is overexpressed in several types of cancers that retained wild-type p53 including gliomas, a number of pre-B acute lymphoblastic leukemias, tumor cell lines, and some primary tumors including breast tumors, head and neck squamous cell carcinomas, and retinoblastomas [24].

3. P53 MUTATIONS

Up until now, more than 2000 different mutants of the p53 have been found, acting in different ways. The mutations found are sporadic, germline, gain-of-function, oncogenic, rebel-angel, Yin and Yang, prion-like, metastasis-inducer, mediator of chemoresistance and modifier of stemness [25].

The somatic mutations in p53 have been reported in almost all the types of human cancers with different prevalence depending on the cancer type. Concerning that, in cancers from the aero-digestive tract (like oral, esophageal or bronchial cancers) the p53 is mutated in 75% of these cases, namely in smokers who are exposed to mutagens. In the case of the cancers in the low digestive tract (such as colon cancer) the p53 mutations are less prevalent being detected in 25% of all the cases. Cancers like cervix, testicular cancers, neuroblastoma and malignant melanomas, present a very low prevalence of p53 mutations (less than 5%). Nevertheless, in these cases, the p53 pathway can be functionally inactivated by viral or cellular oncogenes. An example is cervical cancer where the viral antigen E6 of the oncogenic types of Human Papilloma Virus binds to the wild-type p53 protein and induces its rapid degradation, thus effectively bypassing the need of an inactivating mutation to remove p53 function [26].

Usually, the majority of the tumour suppressor genes like RB, APC, or BRCA1 are inactivated by deletions or truncating mutations during the cancer progression. With regard to p53 mutations in human cancers, they are based on missense mutations in which a single nucleotide is substituted by another [27]. These mutations in p53 are very different and can

be located in different places of its sequence, inducing alterations on its thermodynamic stability. The majority of these mutations are responsible for the loss of p53 ability to bind DNA in a sequence-specific manner and activate transcription of canonical p53 target genes. The higher predominance of mutations in the p53 gene occurs between exons 4-9 that are responsible for encoding the DNA-binding domain of the protein. Among these mutations, 30% fall within 6 "hotspots" residues like R175, G245, R248, R249, R273, and R282 that are very frequent in almost every kind of cancers [28]. The existence of these hotspots could be explained by the susceptibility of particular codons to carcinogen-induced alterations and by positive selection of mutations that render the cell with growth and survival advantages [29]. Furthermore, the loss of function promoted by the mutations in p53 could be responsible for promoting tumour development. In case of a heterozygous situation (where both wildtype and mutant alleles exist) mutant p53 can antagonise wild-type p53 tumour suppressor functions in a dominant negative manner. Also, the inactivation of the wild-type p53 by the mutant p53 in a dominant negative mechanism stems from the fact that the transcriptional activity of wild-type p53 relies on the formation of tetramers, whose DNA binding function may be interfered by mutant p53 [29]. It is also important to consider that during cancer progression p53 mutations are frequently followed by loss of heterozygosity. For example, this kind of loss in the short arm of the chromosome 17, where p53 is located, implies a selective force driving the inactivation of the remaining wild-type allele, suggesting that the dominant negative activity of mutant p53 is not sufficient to completely inactivate wild-type p53 [30]. Moreover, different mutants of p53 isoforms can promote oncogenic activity by a gain-of-function mechanism where there is an acquisition of oncogenic properties by the mutant protein, compared with the mere inactivation of the protein. Regarding all above, dominant negative effect and gain-of-function can promote a selective selection of missense mutations in p53 during tumorigenesis [31].

4. P53-BASED THERAPIES

Nowadays, the most commonly used therapies for cancer treatment are based in chemo or radiotherapy. However, these treatments are usually ineffective to eradicate the tumour and can be extremely influenced by the presence of the wild-type p53, being extremely important to guarantee the regular behaviour of this tumour suppressor gene [32]. The resistance of cancer cells to drugs is a huge drawback in the cancer treatment, being mainly associated to the problems involving drug uptake or export, the prodrug activation or drug inactivation, changes in molecular targets, as well as alterations in DNA repair or modifications in the pro- and anti-apoptotic balance [33].

The p53 direct or indirect influence in cell resistance is dependent on several parameters including the way of action of the drug, the genetic alteration during carcinogenesis and also the cancer cell type. Furthermore, the mutations in p53 promote changes in the proapoptotic balance which by itself promote cancer cell resistance. In order to target tumours that overexpress the mutant p53, specific drugs such as PRIMA-1 (Proline-rich membrane anchor) and PRIMA-1Met have been applied [34]. The mechanism of action of these drugs is not completely understood however is thought that they upregulate p53 target genes such as BAX (Bcl-2-associated X protein), PUMA (p53 upregulated modulator of apoptosis) and NOXA (Phorbol-12-myristate-13-acetate-induced protein 1) rescuing the activity of numerous p53 mutant species [35].

In fact, the influence of p53 in chemotherapy is so important that there are drugs, such as paclitaxel or vincristine, that are responsible for stabilizing wild-type p53 through the inhibition of the transcription associated with the mitotic arrest, preventing the p53 degradation and also affecting the microtubule-mediated transport of p53 [36]. In addition, the drugs used in chemotherapy have similar signaling cascades to the ones involved in p53 activation by DNA damage, and wild-type p53 is required for cell death in tumours after drug exposure [32].

The behaviour of p53 can also be balanced by the regulation of other biomolecules that directly influence its performance. An easy and effective example of this is the good results achieved in cell division control and in the adjustment of p53 levels when drugs that regulate p53-MDM2 protein interaction are applied. Three of the most important molecules responsible for blocking the interaction between these two proteins are small molecules such as nutlins, benzodiazepinediones and spiro-oxindoles [37-39]. Basically, the way of action of these drugs consists in mimicking and inhibit the p53 binding pockets with MDM2 inducing the accumulation of p53 restoring it transcriptional activity followed by apoptosis in MDM2 overexpressing tumour cells [35].

Studies with the application of ionizing radiation showed that the p53 behaviour affects the radiosensitivity of the cells so, concerning that, several studies have been performed using synergistically radiotherapy with gene therapy. For example, Lowe and colleagues (1994) showed that wild-type p53 mouse embryo fibroblasts transformed with the oncogene Hras responded to DNA-damage inducing apoptosis when a 7 gray absorbed dose of ionizing radiation was applied [40]. Koom and collaborators (2012) also studied the effect of the delivery of p53 using an adenoviral vector combined with radiotherapy in hepatocellular carcinoma cells. They showed, that p53 gene transfer using an adenoviral vector would enhance the cellular response to radiotherapy by inhibiting the p53-MDM2 interaction [41]. In addition, non-viral vectors like liposomes containing p53-encoding gene had been used in combination with radiation enabling complete tumour regression and inhibition of their long-term recurrence [42, 43].

The under development therapies to fight the proliferation of cancer cells and progression of tumours, involving the p53 or the p53 pathway as a leading element, still present a lot of problems that limit the generalized clinical application. Regarding that, there are a lot of questions concerning the effectiveness of the therapies involving the p53 that remained to be answered. Although, across the years, the scientists have improved information in this field and have specified and improved therapies trying to make them more personalized, they have studied each cancer particularly in order to find best ways to fight it.

4.1. p53 Reestablishment

Regarding the extreme importance of the expression, integrity and regulation of p53 in the organism for tumour control and to avoid therapy resistance, different therapeutics have been investigated, expecting the targeting of this biomolecule. Gene therapy to restore the p53 levels, inhibition of p53-MDM2 interaction, restoration of mutant p53 to wild-type p53, targeting the p53 family proteins, elimination of mutant p53 and also the development of p53 vaccines are some examples of these alternative strategies [44].

There are several problems concerning the nucleic acids delivery into the cell. Regarding that, one of the main concerns is related to physiological barriers that can change the cellular biodistribution and the intracellular bioavailability. When naked DNA is delivered, it only has the ability to resist degradation during 5 minutes due to its hydrophilic nature and high molecular weight. DNA itself presents a low cellular uptake. Moreover, the DNA that can actually enter into the cell is internalized in vesicles (like endosomes) that can digest DNA, limiting the access to cytoplasmic or nuclear targets. This also represents an important barrier in the gene-based drug efficiency [45].

Gene expression is highly dependent on the access of DNA to the nuclear compartment, which implies successful trafficking to the nucleus and penetration of the nuclear membrane. The nuclear membrane is not highly permeable however, it contains nuclear pore complexes with approximately 25 nm, which are responsible for the export and import of specific molecules to the nucleus. Due to these characteristics, molecules smaller than 40 kDa or particles with a threshold of 25 nm can be diffused through the nuclear pores, whereas larger molecules cannot access to the nucleus.

To overcome these barriers, several delivery systems for p53 gene-based therapeutics have been already successfully developed using different protocols and vectors, including adenovirus, retrovirus, vaccine-derived vectors and different types of nanoparticles modified or non-modified with specific ligands [6].

4.2. Strategies, Advantages and Constraints in the Production of a Clinical Grade Purity p53 Encoding DNA Plasmid

To accomplish the preparation of the genetic material complying with the quality standards for therapeutic application, some biotechnological processes have been developed, based on the design and construction of p53-encoding vectors that can be produced in large scale using recombinant hosts (Fig. **4**). In order to produce the p53 encoding plasmid, a recombinant *Escherichia coli* (*E. coli*) host can be used and grown through suitable fermentation conditions. The biosynthesis of pDNA in these cells enables the production of extracts rich in supercoiled (sc) pDNA, which is advantageous since this pDNA conformation has proved to be more efficient for cells transfection and gene expression than other isoforms, such as the open circular pDNA [46]. However, when alkaline lysis is performed to recover sc pDNA from the recombinant host, high concentrations of other impurities are also released, that must be removed in order to have a final plasmid product with high purity and activity, fulfilling the quality parameters established by the regulatory agencies, such as Food and Drug Administration (FDA), U.S.A.. Concerning that, several specifications are documented, namely regarding the product appearance (clear, colourless solution), the plasmid homogeneity (≈ 97% sc), and impurities levels (RNA and proteins should be undetectable, the amount of genomic DNA must be lower than 2 ng/μg of pDNA and the level of endotoxins should not be higher than 0.1 EU/μg of pDNA) [47, 48]. To guarantee the high level of purity for pDNA, different chromatographic methodologies have been investigated namely, size exclusion, anion exchange, hydrophobic interaction, reversed phase, thiophilic adsorption and, affinity chromatography [49].

Regarding the methods previously mentioned, the affinity approach has been one of the most exploited in the last years, as it has been demonstrated that these methods can specifically purify the most biologically active plasmid conformation. Actually, different studies already proved that a purified sc isoform of p53-encoding pDNA is the most efficient and effective isoform at inducing transgene expression [50]. As an example, in a previous study, Gaspar and colleagues obtained highly pure biopharmaceutical formulations of a p53-encoding vector, by using a strategy where the pcDNA3–FLAG–p53 plasmid was first amplified in a bacterial cell culture of *E. coli* DH5α. Then the authors used L-arginine as a specific ligand for affinity chromatography, and successfully isolated the sc isoform of different plasmids namely the pcDNA3-FLAG-p53 [51]. Completed the plasmid production and purification, the next concern is related with the delivery of the vector to the cells or tissues.

4.3. Viral *vs* Non-viral Vectors

4.3.1. Viral Vectors

Since p53 is lost or mutated in a wide number of cancers, it seems reasonable to try the re-establishment of the p53 expression and function through the replacement of the mutant form by a functional wild-type copy of the gene. One of the strategies used to deliver the correct genetic information is based on the use of suitable viral vectors, like retrovirus or adenovirus. However, the most used viral vectors at the moment are the adenoviral vectors (Adp53) and oncolytic adenoviruses (CRAdp) [26, 52]. The procedures already established attempt to regulate p53 levels in cancer cells. Actually, the activation of p53 can also play an important role in chemosensitizer or in chemoprotective mechanisms, depending on the cellular context. Regarding this, several viral vectors have been produced in order to promote a gene replacement therapy for p53.

In October 2003, the State Food and Drug Administration (SFDA) of China approved type 5 Ad bearing the human wild-type p53 gene (Ad-p53) for the treatment of head and neck cancer [53]. Ad-p53 (Gendicine®, Shenzhen SiBiono GeneTech, Shenzhen, China - now incorporated into Benda Pharmaceutical, Wuhan, China) has been initially developed by Introgen Therapeutics (Advexin®, Austin, TX, USA) for head, neck, and lung cancer treatment. While Introgen was working to obtain US FDA approval, SiBiono successfully completed clinical studies and launched the product. In November 2005, SFDA also approved type 5 Ad defective of