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Selective Induction of Apoptosis in Hepatocellular Carcinoma Cells by AFP Promoter-Driven Apoptin Expression: An *In Vitro* Gene Therapy Approach

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Abstract

Background: Hepatocellular Carcinoma (HCC) remains a leading cause of cancer-related mortality worldwide, and developing tumor-selective therapies with minimal off-target toxicity is a major challenge. Alpha-fetoprotein (AFP), which is highly expressed in HCC but absent in most normal adult hepatocytes, is a promising target for selective gene therapy. Apoptin, a viral protein, induces apoptosis preferentially in malignant cells.

Methods: An AFP promoter-driven Apoptin expression construct was generated using the pAdenoVator-IRES-GFP vector and transiently transfected into HepG2 cells. Promoter specificity was evaluated in AFP-negative HeLa and LNCaP cells. Transfection efficiency was assessed by GFP fluorescence microscopy. Cell viability was measured by MTT assay, Apoptin mRNA expression by RT-qPCR, and apoptosis by Annexin V/7-AAD flow cytometry. Experiments were performed in triplicate.

Results: GFP fluorescence was visibly stronger in HepG2 cells than in HeLa or LNCaP cells on qualitative microscopic examination, consistent with AFP promoter selectivity. AFP promoter-driven Apoptin expression significantly reduced HepG2 cell viability to 50.2%±4.3% of control levels ($p=0.0317$, $n=3$ independent experiments). RT-qPCR showed a significant increase in Apoptin mRNA expression in transfected HepG2 cells. Flow cytometry showed a significant increase in apoptosis, with approximately 20% more Annexin V-positive cells than controls ($p=0.0286$, $n=3$ independent experiments).

Conclusion: AFP promoter-driven Apoptin expression selectively induced apoptosis and reduced viability in HCC cells *in vitro*. These findings highlight the potential of AFP promoter-based Apoptin gene therapy as a tumor-selective therapeutic approach for HCC and support further evaluation in preclinical models.

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Introduction

Hepatocellular Carcinoma (HCC) is the most common primary liver malignancy, accounting for approximately 80% of liver cancer cases worldwide, and remains a leading cause of cancer-related mortality. Despite recent advances in systemic therapies, the prognosis of patients with advanced HCC remains poor, highlighting the need for more effective and tumor-selective therapeutic approaches. In particular, patients who are not candidates for liver-directed therapies continue to face limited treatment options and unfavorable survival outcomes [1,2].

Gene therapy has emerged as a promising therapeutic strategy by enabling the delivery of therapeutic genes directly into hepatocytes or HCC cells using engineered viral vectors [3]. Such approaches aim to selectively modulate oncogenic signaling pathways, thereby improving therapeutic precision while reducing systemic toxicity. However, a significant limitation of current gene therapy strategies is the lack of tumor specificity associated with widely used constitutive

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promoters, such as the Cytomegalovirus (CMV) promoter. Non-selective promoter activity may result in off-target gene expression and unintended adverse effects in normal tissues [4].

Recent work continues to focus on engineering liver-specific and tumor-selective promoters to improve the strength and specificity of liver-directed gene therapy, alongside broader efforts to translate such vector and promoter designs from preclinical models toward clinical application [5,6].

Alpha-fetoprotein (AFP), a well-established biomarker for hepatocellular carcinoma, represents an attractive target for tumor-specific therapeutic applications. AFP expression is highly active during fetal liver development but is largely silenced in normal adult hepatocytes. In contrast, AFP expression is aberrantly reactivated in HCC and is frequently associated with tumor progression and advanced disease stages [5,6]. This tumor-specific reactivation of AFP transcription provides a unique opportunity to develop targeted gene therapy approaches selectively targeted toward AFP-positive HCC cells. Apoptin is a viral-derived pro-apoptotic protein originating from the chicken anemia virus and has attracted considerable interest due to its selective cytotoxicity toward malignant cells while sparing normal cells [7].

The Apoptin protein, encoded by the *VP3* gene of the Chicken Anemia Virus (CAV), consists of 121 amino acids and shows no significant sequence homology to known cellular proteins. Initially identified as an inducer of apoptosis in chicken thymus and lymphoblastoid cell lines, Apoptin has since been recognized as a potent tumor-selective apoptotic agent [8].

Notably, Apoptin has been shown to induce apoptosis in a wide range of human cancer cell types, including melanoma, hepatoma, lymphoma, cholangiocarcinoma, intestinal carcinoma, lung cancer, and prostate cancer [9]. Importantly, the tumor-selective activity of Apoptin appears to be largely independent of the mutational status of major tumor suppressor genes, making it an attractive candidate for targeted cancer gene therapy. Interest in Apoptin-based oncolytic and gene-directed strategies for hepatocellular carcinoma remains active, with recent studies continuing to demonstrate potent antitumor, pro-apoptotic effects of Apoptin-expressing constructs in HCC and other cancer cell lines [10,11].

Mechanistically, Apoptin-mediated apoptosis primarily occurs through activation of the intrinsic mitochondrial pathway in transformed cells, while normal cells remain largely unaffected. Apoptin promotes the release of cytochrome c (Cyto-C) and Apoptosis-Inducing Factor (AIF), leading to mitochondrial membrane potential disruption and apoptotic cell death [12]. Importantly, Apoptin-induced apoptosis is independent of p53 status, as both p53-positive and p53-deficient cancer cells exhibit comparable susceptibility to Apoptin-mediated cytotoxicity [13].

In addition to mitochondrial disruption, Apoptin activates downstream caspase signaling cascades. Unlike extrinsic apoptosis pathways, Apoptin-induced cell death does not rely on death receptors such as CD95, caspase-2, or FADD. Instead, Apoptin facilitates mitochondrial cytochrome c release, leading to caspase-9 activation and subsequent apoptotic execution [14].

Despite progress in systemic therapies, including tyrosine kinase inhibitors and immune checkpoint inhibitors, current treatment strategies for advanced HCC remain limited by systemic toxicity, the development of therapeutic resistance, and off-target effects resulting from their broad mechanisms of action [14,15].

Although AFP promoter-regulated Apoptin systems have been investigated for hepatocellular carcinoma, most studies have relied on replicative viral systems or have not comprehensively evaluated promoter specificity in AFP-negative tumor cell lines. Furthermore, information on the performance of non-replicative AFP promoter-driven Apoptin expression systems is limited. Therefore, the present study was designed to construct and evaluate a non-replicative AFP promoter-driven Apoptin expression vector and to investigate its selective apoptotic activity in AFP-positive hepatocellular carcinoma cells while assessing promoter specificity in AFP-negative cancer cell lines. Authors have hypothesized that AFP promoter-driven Apoptin expression would selectively induce apoptosis in AFP-positive hepatocellular carcinoma cells while minimizing transgene expression in AFP-negative cells.

The originality and significance of the present study rest on three features that distinguish it from prior AFP promoter-Apoptin research. First, unlike the replicative oncolytic adenoviral system reported by Zhang *et al* [16], the present study's construct is non-replicative, avoiding the safety concerns associated with uncontrolled viral propagation while still achieving robust, tumor-restricted Apoptin expression. Second, the authors have directly assessed AFP promoter selectivity by qualitative fluorescence microscopy, not only in AFP-positive HepG2 cells but also in two independent AFP-negative cell lines of distinct tissue origin (HeLa and LNCaP), a comparison that has not been comprehensively addressed in earlier non-replicative systems. Third, this study integrates promoter-level (GFP reporter), transcript-level (RT-qPCR), and functional (MTT viability and Annexin V/7-AAD apoptosis) evidence within a single platform, providing converging, multi-level confirmation of tumor-selective activity. Together, these features position the AFP promoter-driven, non-replicative Apoptin system as a safer and more rigorously validated proof-of-concept platform for tumor-selective HCC gene therapy, addressing a gap left by earlier replicative or less comprehensively characterized approaches.

Materials and Methods

Virus and genomic DNA template

A live attenuated Chicken Anemia Virus (CAV) vaccine strain was obtained from the Faculty of Veterinary Medicine, Tehran University, Iran, and used solely as a DNA source for amplification of the apoptin gene. The vaccine strain was used exclusively as a molecular template for gene amplification and was not employed in any infection or biological activity experiments. The apoptin protein is encoded by the *VP3* gene of CAV. The *VP3* gene encoding Apoptin (366 *bp*) was selected for amplification and cloning.

Isolation and amplification of the apoptin gene

Total genomic DNA was extracted from the CAV-VAC isolate using the Accuprep Genomic DNA Kit (Bioneer, Korea) according to the manufacturer's instructions. Specific primers incorporating *Xba*I and *Bgl*II restriction sites at the 5' ends were designed to amplify the full-length apoptin gene. PCR amplification was performed using AccuPower PCR PreMix (Bioneer, Daejeon, Korea) according to the manufacturer's instructions, with an initial denaturation at 95°*C* for 5 *min*, followed by 33 cycles of denaturation at 95°*C* for 30 *s*, annealing at 53°*C* for 30 *s*, and extension at 72°*C* for 45 *s*, with a final extension at 72°*C* for 5 *min*.

PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Germany) and sequenced using an ABI PRISM 3730 DNA Analyzer. Sequence identity was confirmed using the NCBI BLAST tool. Sequencing results showed 100% identity to the reference apoptin (VP3) sequence in the GenBank database.

Construction of the AFP promoter-driven Apoptin expression plasmid

The amplified apoptin gene and the pAdenoVator CMV-IRES-GFP plasmid backbone were digested with *Xba*I and *Bgl*II, then ligated to generate the recombinant expression construct. The ligation products were transformed into *Escherichia coli* DH5α competent cells and plated on LB agar containing kanamycin. Colony PCR screened positive clones, verified them using the AccuPrep® plasmid Mini Extraction Kit, and used them for subsequent transfection experiments. Independent cloning experiments were repeated at least twice to confirm reproducibility.

Extraction and amplification of the AFP promoter and enhancer

Genomic DNA was extracted from the human hepatoma cell line HepG2 to amplify the AFP promoter and enhancer regions. PCR amplification was performed using primers listed in table 1. Cycling conditions included an initial denaturation at 95°*C* for 5 *min*, followed by 35 cycles of denaturation, annealing, and extension. The AFP promoter was amplified at 52°*C*, while a second round of enhancer amplification was performed at 58°*C* to improve specificity. Restriction sites for *SSP*1 and *Sal*1 enzymes were incorporated during the second amplification step.

Linking the AFP Promoter to the Enhancer Using SOE-PCR

Splicing by overlap extension PCR (SOE-PCR) was employed to fuse the 94 *bp* AFP promoter with the 75 *bp* enhancer fragment. Initially, 12 PCR cycles were performed without primers, followed by the addition of forward and reverse primers and 23 additional cycles. The resulting 1111 *bp* fusion fragment was confirmed by electrophoresis on a 1% agarose gel, purified, and stored for further cloning. The resulting fusion fragment included the AFP promoter and enhancer regions together with adjacent regulatory flanking sequences, which contributed to the larger final fragment size.

Cloning of the AFP promoter/enhancer region into the Apoptin expression plasmid

The pAdenoVator-CMV-Apoptin-IRES-GFP plasmid vector and the AFP promoter/enhancer fragment were digested with *Sal*I and *Xba*I enzymes and purified. The promoter/enhancer fragment was subsequently cloned upstream of the apoptin gene to generate the pAdenoVator-AFP-Apoptin-IRES-GFP construct for functional evaluation.

Cell line and transfection

The HepG2 cell line (ATCC HB-8065) was authenticated by the supplier. All cell lines were routinely tested for mycoplasma contamination using an in-house PCR-based detection method and were confirmed to be mycoplasma-free before experimentation. The HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicil-

Table 1. Primer sequences used for amplification of apoptin and AFP promoter/enhancer regions

Target gene	Primer type	Sequence (5′–3′)	Product size (*bp*)
Apoptin	Forward	CTAG**TCTAG**AATGAACGCTCTCCAAGAAGAT	389
Apoptin	Reverse	AATGCGATCGTTAC**AGTCT**TATACGCCTTTTTG	389
AFP promoter	Forward	TTGAAGTCGACAGTTTGAGGAGAATATTTGTT	294
AFP promoter	Reverse	CTTTATTCTAGACCCCATGGTTGCTAGTTATTTTGTTATTG	294
AFP enhancer (1st round)	Forward	ACTAACCAACCACCCAATCCAAC	1070
AFP enhancer (1st round)	Reverse	CAATGCACACTTAGGGAATGAGAGA	1070
AFP enhancer (2nd round)	Forward	GACGC**AATATT**AAATTAGTTTTGAATCTTTCT	725
AFP enhancer (2nd round)	Reverse	ATTAT**GTCG**ACGGATAAAGCTGAGTGGTATG	725

Restriction enzyme sites are shown in bold and underlined.

lin/streptomycin at 37°*C* in a humidified 5% CO_2 atmosphere.

For the transfection experiments, HepG2 cells were seeded at a density of 6.2×10^4 cells per well in a 6-well plate and transfected with the pAdenoVator-AFP-Apoptin-IRES-GFP construct using Lipofectamine 3000 (Invitrogen, USA). For each well, 2 μg plasmid DNA and 5 μl Lipofectamine 3000 reagent were used according to the manufacturer's instructions. AFP-vector control-transfected cells treated with Lipofectamine 3000 in the absence of plasmid DNA were used as negative controls. Seventy-two *hr* post-transfection, GFP expression was evaluated by fluorescence microscopy to assess transfection efficiency. At least five randomly selected microscopic fields were evaluated for each experimental condition to estimate transfection efficiency. To evaluate promoter specificity, LNCaP (prostate carcinoma) and HeLa (cervical carcinoma) cell lines were transfected with the same constructs and analyzed for GFP expression. LNCaP and HeLa cell lines were similarly authenticated and confirmed to be free of mycoplasma before use.

Transfection efficiency was calculated using the following formula:

Transfection efficiency (%)=(Number of GFP-positive cells/Total number of cells)×100

All transfection experiments were independently repeated three times.

Functional assessment of HepG2-specific promoter

A schematic representation of the cloning strategy (Figure S1) was included to illustrate the stepwise assembly of the AFP promoter-enhancer region and its ligation to the apoptin gene within the pAdenoVator vector.

MTT assay

Cell viability was assessed using the MTT assay. HepG2 cells were seeded at a density of 5×10^3 cells per well in a 96-well plate. After 72 *hr* of transfection, 20 μl of MTT solution was added to each well, and the wells were incubated for 4 *hr* at 37°*C*. Formazan crystals were dissolved in 100 μl of DMSO, and the absorbance was measured at 490 *nm*. Cell viability was expressed as a percentage relative to the AFP-vector control-transfected control group, which was set to 100% viability.

Quantitative real-time PCR analysis

Total RNA was extracted 72 *hr* post-transfection using AccuZol™ reagent (Bioneer, Daejeon, Korea). cDNA synthesis and quantitative real-time PCR (RT-qPCR) were performed using SYBR Green Master Mix (Takara Bio Inc., Shiga, Japan). Cq values were obtained using the Rotor-Gene 5 Plus real-time PCR system with HRM capability (Qiagen, Hilden, Germany) and analyzed with Rotor-Gene software version 2.0.2.4. Relative gene expression was calculated using the 2^-ΔΔCq method [15]. Amplification specificity was confirmed by melt curve analysis. β-actin was used as the internal reference gene due to its stable expression across experimental conditions. All reactions were performed in triplicate, and no-template controls were included to exclude contamination and non-specific amplification.

Apoptosis analysis

Apoptosis was evaluated using PE-Annexin V and 7-AAD staining. HepG2 cells were seeded at a density of 1×10^6 cells per well in 6-well plates and transfected as described above. After 48 and 72 *hr*, cells were harvested, washed, and stained according to the manufacturer's protocol. Samples were analyzed using a BD FACS Calibur flow cytometer (BD Biosciences, USA), and the data were processed with FlowJo 2.6 software (Tree Star Inc., USA). A minimum of 10,000 events were acquired for each sample. Apoptotic cells were identified as Annexin V-positive populations, including both early and late apoptotic fractions.

Statistical analysis

Statistical analyses were performed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA). Data are presented as mean±standard deviation (SD) from three independent experiments. Comparisons between two groups were performed using the unpaired Student's t-test. The normality of the data distribution was assessed before analysis. A p-value <0.05 was considered statistically significant.

Results

Isolation, amplification, and cloning of the apoptin gene

Total genomic DNA was successfully extracted from the CAV-VAC isolate and used as a template to amplify the full-length apoptin gene. PCR amplification under optimized conditions consistently yielded the expected-size PCR product. Sequencing confirmed complete sequence fidelity of the apoptin gene, with no mutations detected. The gene was then successfully cloned into the pAdenoVator CMV-IRES-GFP vector, with colony PCR and sequencing validating the correct orientation and integrity of the insert. Purified recombinant plasmid DNA was prepared for subsequent transfection experiments.

Amplification of the AFP promoter/enhancer and integration into the apoptin expression plasmid

DNA extracted from the HepG2 cell line enabled the successful amplification of both the AFP promoter and enhancer sequences. Sequencing analysis confirmed the identity of the amplified AFP promoter/enhancer fragments and verified the absence of sequence alterations introduced during PCR amplification.

The production of a 294 *bp* promoter fragment was confirmed, along with the generation of a 1070 *bp* enhancer fragment through PCR using specific primers. A subsequent PCR reaction using a different set of enhancer primers produced a distinct 725 *bp* fragment. These sequences were effectively fused using the Splicing by Overlap Extension PCR (SOE-PCR) meth-

od, yielding a purified fragment. Additionally, a 1000 *bp* DNA fragment was amplified using reverse promoter and forward enhancer primers and verified by sequencing. This fragment was then cloned into the Apoptin expression plasmid, and restriction digestion confirmed the successful insertion. The recombinant plasmid construct was subsequently used for functional studies.

Confirmation of transfection efficiency using the pAdenoVator-CMV5-IRES-GFP construct across cell lines

HepG2, LNCaP, and HeLa cells were transfected with the pAdenoVator-CMV5-IRES-GFP construct using Lipofectamine 3000, according to the manufacturer's protocol. Cells were seeded at a density of 62,000 cells per well in 6-well plates and incubated for 24 *hr* to reach approximately 75% confluency before transfection. Transfection efficiency was evaluated 48 *hr* post-transfection using fluorescence microscopy to assess GFP expression alongside bright-field imaging of cell morphology (Figure 1). Robust GFP expression was observed in all three cell lines, consistent with the ubiquitous activity of the CMV5 promoter, confirming successful transfection and validating the delivery method before use with tissue-specific constructs.

In contrast, AFP-negative LNCaP and HeLa cells transfected with the AFP promoter-driven construct showed minimal or absent GFP expression. Transfection efficiency was evaluated 72 *hr* post-transfection using fluorescence microscopy to assess GFP expression in the transfected cells (Figure 2).

The transfection efficiency was quantified by counting GFP-positive cells with clear nuclei relative to the total number of cells in each microscopic field. The results suggest that the liver-specific promoter driving the apoptin gene in the experimental construct effectively drives transgene expression in HepG2 cells, resulting in significant GFP expression. In contrast, AFP-negative LNCaP and HeLa cells transfected with the AFP promoter-driven construct showed minimal or absent GFP expression. These findings are consistent with liver cancer-selective transcriptional activity of the AFP promoter, based on qualitative observation. The absence of detectable GFP expression in AFP-negative cell lines is consistent with specificity of AFP

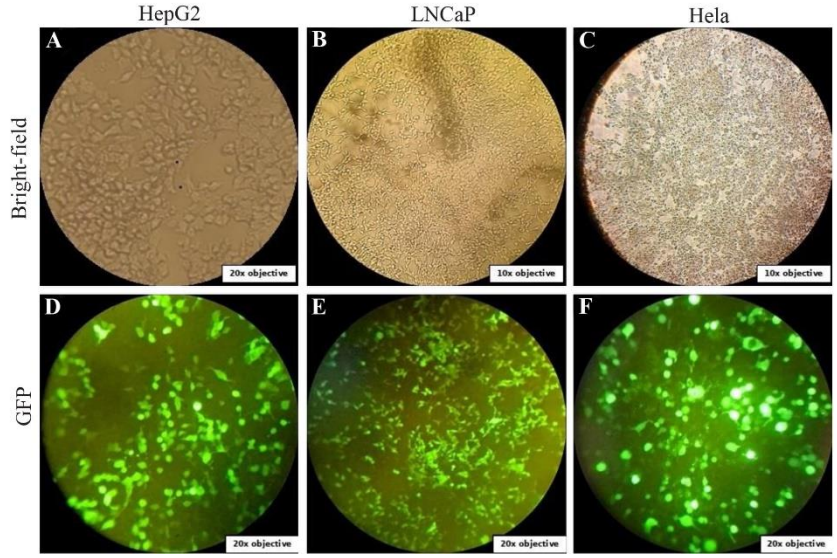

Figure 1. Bright-field and GFP fluorescence images of HepG2, LNCaP, and HeLa cells transfected with pAdenoVator-CMV5-IRES-GFP, showing successful transfection across all three cell lines.
A, D) HepG2: bright-field (20x) and GFP (20×), B, E : LNCaP: bright-field (10x) and GFP (20x), C, F : HeLa: bright-field (10×) and GFP (20x)

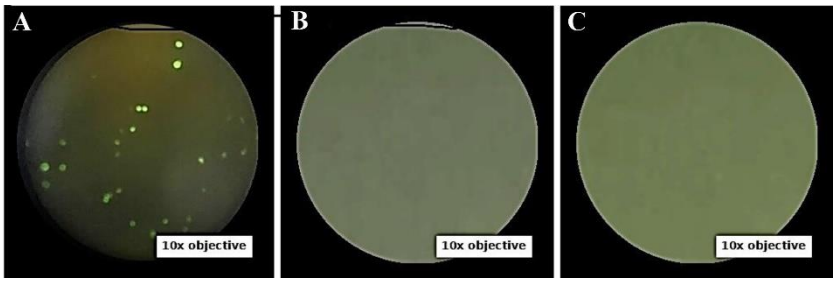

Figure 2. Transfected cells with the pAdenoVator-AFP-Apoptin-IRES-GFP construct after 48 *hr*.
A) HepG2 cells show clear GFP expression, indicating active AFP promoter functionality in hepatocellular carcinoma cells.
B) LNCaP cells exhibit no GFP expression, consistent with a lack of AFP promoter activity in non-hepatic prostate cancer cells (qualitative assessment).
C) HeLa cells also show no GFP expression, consistent with AFP promoter selectivity for hepatic cells (qualitative assessment).

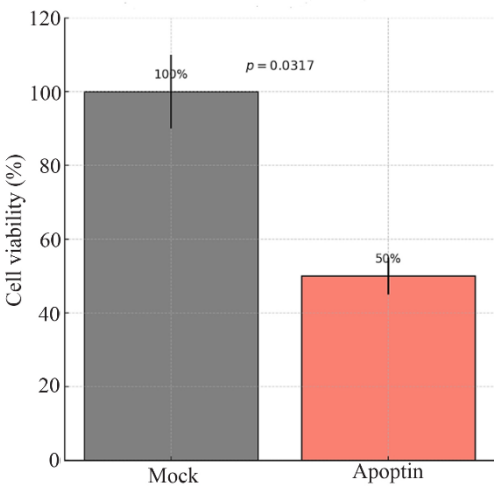

Figure 3. Effect of AFP promoter-driven Apoptin expression on HepG2 cell viability. The Apoptin-expressing group showed significantly reduced cell viability compared with AFP-vector control-transfected controls (p=0.0317).

promoter-mediated transcriptional regulation; however, fluorescence intensity and the percentage of GFP-positive cells were not quantified or statistically compared among cell lines in this study, and these findings should therefore be interpreted as qualitative. It is important to note that GFP expression in the IRES-based construct indicates successful transcription and transfection efficiency but does not necessarily reflect the expression level or function of the upstream Apoptin gene. Therefore, GFP-positive cells confirm vector uptake and promoter activity, while apoptin gene expression and its apoptotic effect were independently validated using real-time PCR and flow cytometry.

Effect of apoptin expression on HepG2 cell viability

The results demonstrated that apoptin expression significantly reduced HepG2 cell viability compared to the control group. Specifically, apoptin-expressing cells exhibited approximately 50% reduced viability compared with AFP-vector control-transfected controls. This reduction was statistically significant (p=p 0.0317), as determined using Student's t-test (Figure 3).

Evaluation of apoptin gene expression using real-time PCR

Real-time PCR was performed to assess Apoptin gene expression in HepG2 cells 72 *hr* post-transfection. The RT-PCR analysis revealed significantly elevated Apoptin mRNA expression levels in cells transfected with the pAdenoVator-AFP-Apoptin-IRES-GFP construct compared to the control group transfected with pAdenoVator-CMV5-IRES-GFP. RT-qPCR analysis demonstrated a significant increase in Apoptin mRNA expression in HepG2 cells transfected with the pAdenoVator-AFP-Apoptin-IRES-GFP construct compared with control cells. These findings confirmed successful AFP promoter-driven transcription of the Apoptin gene in HCC cells.

Analysis of apoptosis in HepG2 cells using flow cytometry

Flow cytometry was employed to evaluate the apoptosis-inducing effects of the pAdenovator-AFP promoter/enhancer-Apoptin-IRES-GFP vector in HepG2 cells. Flow cytometric analysis demonstrated a significant increase in Annexin V-positive apoptotic cells in the Apoptin-expressing group compared with the control group (p=0.0286) (Figure 4).

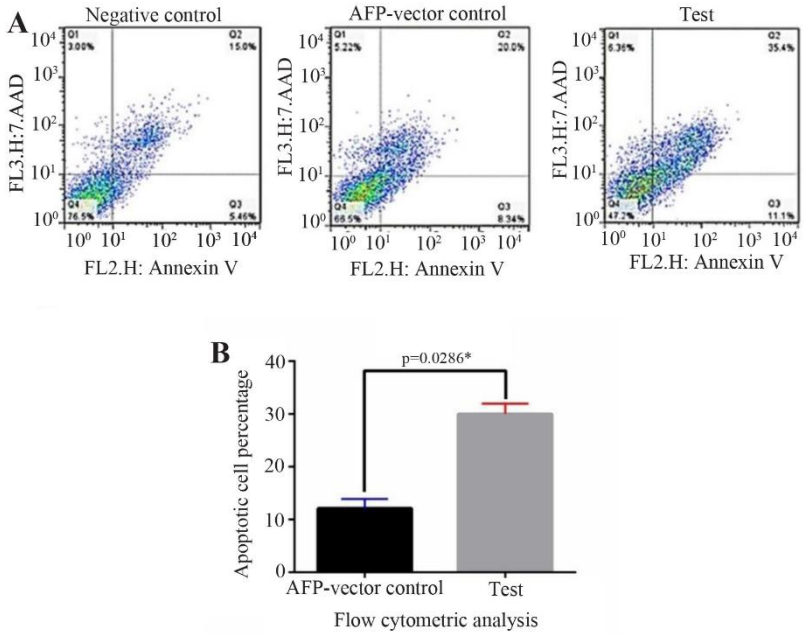

Figure 4. Flow cytometric analysis of Apoptin-induced apoptosis in HepG2 cells. Flow cytometry was performed in three independent experiments (n=3). A) Representative dot plots of Annexin V/7-AAD staining. Negative control: HepG2 cells transfected without any plasmid vector. AFP-vector control: HepG2 cells transfected with the pAdenovator-AFP-IRES-GFP vector, representing baseline apoptosis levels. Test: HepG2 cells expressing Apoptin via the pAdenovator-AFP promoter/enhancer-Apoptin-IRES-GFP vector. B) Bar graph summarizing the mean apoptotic (Annexin V-positive) cell percentage for AFP-vector control and Test groups across the three experiments (error bars, SD). Apoptin expression significantly increased the apoptotic cell percentage relative to AFP-vector control (p=0.0286).

Discussion

This study describes a targeted gene therapy strategy for HCC that selectively expresses Apoptin under the control of the AFP promoter in AFP-positive liver cancer cells. The findings of the present study demonstrate that combining a tumor-specific promoter with a tumor-selective pro-apoptotic gene can achieve targeted gene expression and induction of apoptosis in HCC cells while minimizing the potential for off-target effects.

A central finding of this study is the effective utilization of the AFP promoter to drive selective gene expression in HCC cells. AFP is transcriptionally active during fetal liver development but remains largely silenced in normal adult hepatocytes. Its reactivation in HCC is well documented and has been shown to correlate with tumor progression and poor prognosis [17-19]. In the present study, AFP promoter activity was clearly demonstrated in HepG2 cells. In contrast, no appreciable transcriptional activity was observed in AFP-negative non-hepatic cancer cell lines on qualitative assessment, consistent with tumor-selective activity of this regulatory element. This selectivity is crucial for gene therapy applications, where uncontrolled transgene expression remains a significant limitation.

Apoptin is recognized for its unique ability to induce apoptosis in malignant cells while sparing normal tissues selectively [9,12,20]. The results of this study further substantiate this property by demonstrating a significant reduction in HepG2 cell viability and a marked increase in apoptotic cell populations following AFP-driven Apoptin expression. The observed apoptotic response is consistent with previous reports showing that Apoptin induces cell death primarily through the intrinsic mitochondrial pathway and acts independently of p53. This p53-independent mechanism is particularly relevant in HCC, where p53 mutations are common and often contribute to resistance to conventional therapies.

The apparent discrepancy between the magnitude of viability loss observed in the MTT assay (~50%) and the proportion of apoptotic cells detected by flow cytometry (~20%) reflects the distinct biological processes assessed by these assays. While the MTT assay captures cumulative metabolic impairment resulting from both apoptotic and non-apoptotic mechanisms, flow cytometry specifically quantifies cells undergoing apoptosis at a defined time point. Accordingly, these findings should be regarded as complementary rather than contradictory and together provide a more comprehensive evaluation of Apoptin-mediated cytotoxicity.

The incorporation of the apoptin gene into a pAdenoVator plasmid construct under the control of the AFP promoter represents a rational strategy for tumor-selective transgene expression in HCC [21,22]. The real-time PCR and flow cytometric data collectively confirm that AFP-driven Apoptin expression results in functionally relevant apoptosis in HCC cells.

The inclusion of both the AFP promoter and enhancer elements ensured robust transcriptional activity while maintaining strict tumor specificity. This was further supported by the qualitative absence of GFP expression in non-hepatic cell lines, suggestive of the safety profile of this regulatory system, although quantitative confirmation is needed. These findings indicate that the AFP promoter demonstrated sufficient transcriptional activity to achieve biologically meaningful levels of Apoptin expression in HCC cells.

Despite the encouraging *in vitro* findings, several challenges must be addressed before clinical translation. Future viral or nanoparticle-based delivery systems may be limited by immune responses, vector dilution, and restricted tumor penetration following systemic administration [23,24]. Moreover, long-term safety and durability of therapeutic efficacy require evaluation in appropriate animal models. These considerations highlight the need to optimize delivery strategies further and the dosing regimen.

Compared with alternative gene therapy approaches for HCC, the Apoptin system offers distinct advantages. Its strong tumor selectivity, combined with a p53-independent apoptotic mechanism, makes it particularly suitable for genetically heterogeneous tumors such as HCC. Nevertheless, plasmid-based delivery systems may face limitations in transfection efficiency and *in vivo* delivery, which can limit repeated administration and underscore the potential benefit of next-generation delivery systems, such as nanoparticle-based carriers or hepatocyte-targeted ligands.

The therapeutic potential of this strategy may be further enhanced through combination approaches. Integrating Apoptin-based gene therapy with existing treatments, including tyrosine kinase inhibitors or immune checkpoint inhibitors, may improve overall treatment efficacy and overcome resistance mechanisms [10]. Such multimodal strategies are particularly relevant given the limited options currently available for patients with advanced HCC.

Finally, when compared with the study by Zhang *et al* [16], which employed a dual-regulated oncolytic adenovirus expressing Apoptin under promoter control, our approach emphasizes safety and transcriptional specificity. Unlike replicative oncolytic vectors that require viral genome modifications and may pose safety concerns, in this study non-replicative adenoviral plasmid backbone minimizes the risk of unintended viral propagation.

Furthermore, the authors qualitatively assessed the AFP promoter's selectivity in AFP-negative cell lines, which is consistent with, but does not by itself establish, its specificity. Collectively, these results support the AFP promoter-Apoptin system as a potentially selective therapeutic strategy for HCC.

This study has several limitations. First, experiments were performed exclusively *in vitro* using a sin-

gle AFP-positive HCC cell line (HepG2); validation in additional AFP-positive HCC lines, such as Huh7 or Hep3B, would strengthen confidence in the generalizability of these findings and should be addressed in future studies. Second, detailed mechanistic analyses of apoptotic signaling pathways were not conducted. Finally, recombinant adenoviral particles were not generated; therefore, the transfection system does not fully replicate *in vivo* gene delivery conditions. Future studies should include animal models and mechanistic investigations to validate the therapeutic potential of this strategy further.

Additional limitations include the absence of direct protein-level confirmation of Apoptin expression (*e.g.*, by Western blotting or immunofluorescence), quantitative image analysis of GFP-based promoter specificity (fluorescence intensity and percentage of GFP-positive cells with statistical comparison across cell lines), dedicated CMV-Apoptin and AFP-empty-vector control arms across all functional assays, and primer efficiency validation with raw Ct-value reporting for RT-qPCR; these will be addressed in follow-up studies.

Conclusion

This *in vitro*, single-cell-line, transient-transfection study provides preliminary proof-of-concept evidence that the AFP promoter can drive tumor-selective Apoptin expression, resulting in significant apoptosis and reduced cell viability in HepG2 cells. These findings should be interpreted as an early-stage feasibility demonstration rather than evidence of therapeutic efficacy; in the absence of animal, xenograft, biodistribution, and toxicity data, any translational or therapeutic potential remains speculative. *In vivo* validation, including biodistribution and toxicity studies in animal models, is required before the therapeutic promise of this approach can be assessed, consistent with broader translational considerations recently outlined for liver-directed gene therapy [6].

Ethics approval and consent to participate

This study did not involve human participants, human tissue samples, or animal subjects. All experiments were performed using commercially available, established human cell lines obtained from the Pasteur Institute Cell Bank. As no primary human or animal material was collected or used in this research, approval from an institutional ethics committee was not required. All experimental procedures complied with the relevant institutional biosafety regulations.

Use of artificial intelligence tools

The authors used generative AI tools solely for English language editing and manuscript refinement. All scientific content, interpretations, and conclusions were independently reviewed and approved by the authors.

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Conflict of Interest

The authors declare no competing interests.

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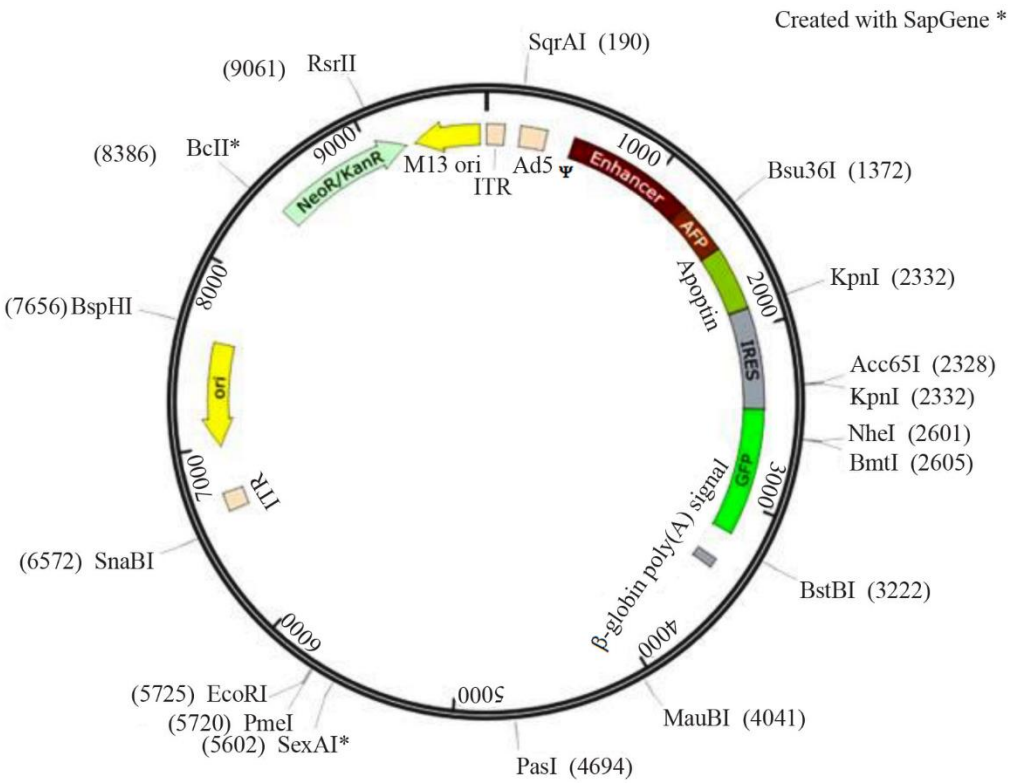

Figure S1. pAdempVatpr-AFP promoter-apoptin-IRES-GFP 9672 *bp*.