



Research Article

Human Gyrovirus Apoptin as a Potential Selective Anticancer Agent: An In Vitro Study

Amir Akbari^{1,2}, Rita Arabsolghar^{1,2*}, Abbas Behzad Behbahani^{1,2}, Gholamreza Rafiei Dehbidi^{1,2}, Farahnaz Zare^{1,2}, Mahdeih Hadi^{1,2}

¹Department of Medical Laboratory Science, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran.

²Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran.

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ABSTRACT

Background: Selective therapy has always been the main challenge in cancer treatments. Recently, it has been shown that Human Gyrovirus-derived protein apoptin (HGV-Apoptin) has selective cytotoxic effects on cancer cells similar to its homologue, Chicken Anemia Virus-derived Apoptin (CAV-Apoptin). However, apoptotic effects of Human Gyrovirus apoptin have been only evaluated on a few cancerous cell lines and need to be further investigated. In this study, we have evaluated the apoptotic effects of HGV-Apoptin and CAV-Apoptin expression on lung cancer (A549) and normal (HEK-293) cell lines, in order to provide more information about the specificity of these proteins on cancerous cells.

Methods: Target cells were transfected by the calcium-phosphate precipitation method with constructed plasmids expressing HGV-Apoptin and CAV-Apoptin proteins as well as the control plasmid. Transfection efficiency was followed and imaged by fluorescence microscopy. Quantification of apoptosis was performed by flow cytometry. Measurements were compared by paired Student t-test.

Results: Cells were successfully transfected with control and constructed plasmids. Flowcytometry analysis showed that A549 cells transfected with HGV-Apoptin and CAV-Apoptin expressing plasmids, undergone the apoptosis compared to A549 cells transfected with control plasmid ($P < 0.001$). None of the plasmids could induce apoptosis in HEK-293 cells.

Conclusion: Human Gyrovirus-derived apoptin (HGV-Apoptin) similar to its homologue, chicken anemia virus derived Apoptin (CAV-Apoptin) can induce apoptosis in Non-small-cell lung carcinoma cell line A549, but not in normal human embryonic kidney cell line HEK-293, which can be introduced as a promising novel specific antitumor agent.

Introduction

Chicken anemia-derived Apoptin is a 13.6 kD protein encoded by VP3 gene of chicken anemia virus (CAV), the first species of the Gyrovirus genus, from Circoviridae family. CAV has a single stranded DNA genome with three partially overlapping open reading frames that encode the VP1, VP2 and VP3 (Apoptin) peptides.¹ To date, it has been shown that the expression of apoptin induces apoptosis in a broad range of cancer cells including hepatoma, osteosarcoma, melanoma, cholangiocarcinoma, colon carcinoma, lung cancer, breast cancer, prostate cancer, cervix cancer, and gastric cancer, while having no effects on cell death of normal cells.^{2,3}

The exact mechanisms of specific apoptosis caused by CAV-Apoptin in cancerous cells are not yet clearly understood. However, based on the existing evidence, the property of Apoptin's tumor-specific killing activity is

the consequence of several molecular events.^{4,6} Apoptin acts through the intrinsic mitochondrial pathway, in a caspase-3 and caspase-9 dependent and p53 independent manner.^{7,8} It is also proposed that Apoptin-mediated cell death involves the modulation of the sphingomyelin-ceramide pathway.⁹ The selective toxicity of apoptin might be attributed to its differential subcellular localization in tumor and normal cells, which is controlled by phosphorylation.^{10,11} Apoptin shows predominantly nuclear localization in tumor cells, but in normal cells, it is mainly detected in cytoplasm.^{12,13}

Recently, human homologue of CAV, named human gyrovirus (HGV), has been identified in a skin swab sample of a healthy person. Organization of HGV genome is similar to CAV, which contains three partially overlapping open reading frames, encoding several proteins.¹⁴ The HGV vp3 (i.e. apoptin) protein has an approximately 40% identity with its homologue in CAV.

*Corresponding Author: Rita Arabsolghar, E-mail: arabsolghar@sums.ac.ir

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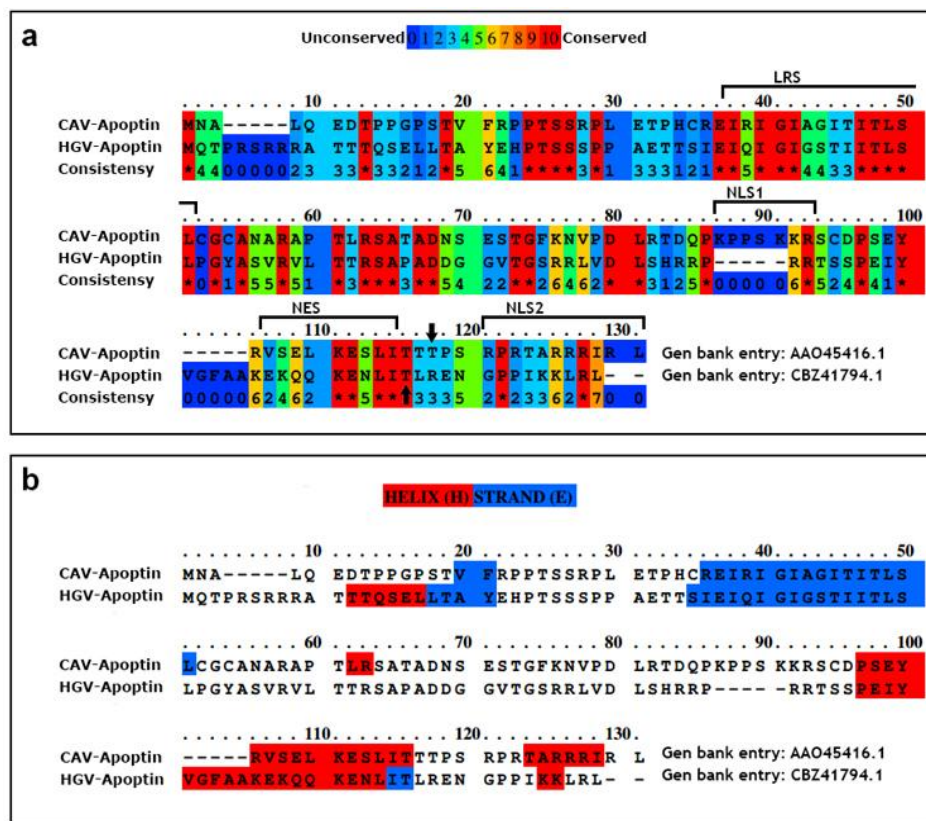


Figure 1. (a) The colorscheme of the alignment between chicken anemia virus-apoptin (CAV-Apoptin) and human gyrovirus-apoptin (HGV-Apoptin) using the conservation scoring performed by PRALINE multiple sequence alignment program.¹⁵ The scoring scheme works from 0, for the least conserved alignment position to 10, for the most conserved alignment position. The color assignment legend is shown in the above figure. The predicted phosphorylation sites threonine 108 or threonine 111 are indicated by black arrows. Abbreviations: LRS, leucine-rich sequence; NES, nuclear export sequence; NLS, nuclear localization sequence. **(b)** The colourscheme of the alignment for secondary structure. The 3-state (H, E, and C) secondary structure for each sequence is represented by a color, which is predicted using DSSP and PSIPRED. If a sequence in the alignment has no color assigned, this means that there is no DSSP and PSIPRED information available.

Despite this insignificant overall identity, sequence conservation in their functional regions, such as nuclear localization and export signals, leucine rich sequence and phosphorylation sites, can be observed (Figure 1a). Furthermore, alignment between secondary structures of HGV-apoptin and CAV-apoptin predicted by DSSP¹⁶ and PSIPRED,¹⁷ indicates that the 3-state secondary structures are placed in similar locations along with two protein sequences (Figure 1b).

So far, studies have shown that HGV-Apoptin can also induce apoptosis in cancer cells in a selective manner and follow the functional mechanisms of CAV-Apoptin to kill the tumor cells. HGV-Apoptin has the same nucleocytoplasmic shuttling as CAV-apoptin. In some tumor cells, it is indicated that HGV-apoptin fused with green fluorescent protein (GFP) translocates to the nucleus, but it is detected mainly in the cytoplasm of normal human fibroblast cells, as has already been observed in CAV-Apoptin.^{10,18} Similar to CAV-Apoptin, HGV-Apoptin triggers apoptosis in a p53-independent manner through the mitochondrial pathway.¹⁹ The rest of functional similarities between these two homologue proteins remain to be determined.

To date, the apoptotic effects of HGV-apoptin are shown only on a few cell lines (HCT116 colon carcinoma, Saos-

2 osteosarcoma, MCF-7 breast cancer cell lines).¹⁸⁻²⁰ In this study, we evaluated the apoptotic effects of this protein on A549 and HEK-293 cell lines and compared it with CAV-Apoptin, in order to provide further evidence regarding the cytotoxicity of HGV-apoptin in cancerous and normal cell lines.

Materials and Methods

Cell lines and cell culture

Non-small-cell lung cancer A549 and human embryonic kidney HEK-293 cell lines (Pasture Institute, Tehran, Iran) were routinely grown in high glucose DMEM medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin. Both cell lines were incubated at 37°C and 5% CO₂.

Synthesis and cloning of genes

To obtain the HGV-apoptin gene sequence, a vector construct including an Ecor.I restriction site preceding a Kozak sequence in the upstream of an ATG start codon, following the HGV-Apoptin sequence gene, and a downstream XbaI restriction site was ordered for synthesis from ShineGene Co. (China). Then, it was cloned in pCMV6-AC-GFP (origene, Washington, US) plasmid.

CAV-Apoptin gene Also was amplified by PCR from commercial vaccines (AviPro THYMOVACTM) and then cloned into pCMV6-AC-GFP. CAV-apoptin gene Amplification for cloning was performed using forward primer: 5'-CTAGGGATCCATGAACGCTCTCCAAGAAGAT-3' and reverse primer: 5' GCTAGTCTAGATTA CAGTCTTATACGCCTTTTTTGC-3' that includes the required restriction sites for subsequent cloning. Cloning was confirmed by DNA Sanger sequencing subsequently. The constructed plasmids were named pHGV-Apoptin-GFP and pCAV-Apoptin-GFP. The control vector used for transfection was pEGFP-C1, which expresses GFP protein without any toxic protein.

Transient transfection

A549 and HEK-293 cell lines were transfected with pHGV-Apoptin-GFP, pCAV-Apoptin-GFP or pEGFP-C1 control vector by calcium-phosphate precipitation as described.²¹ Briefly, exponentially growing cells were seeded at a density of 1×10⁵ to 4×10⁵ cells/cm² in 6-well plates in an appropriate complete medium 24 hours before transfection. One hour before transfection, the medium was renewed. To prepare calcium phosphate-DNA coprecipitate, 27 µl of 2.5 M CaCl₂ with 15 µg of plasmid DNA were combined in a sterile 5-ml plastic tube. Then, the final volume was brought to 400 µl using 0.1X TE (pH 7.6). One volume of the solution was mixed with an equal volume of 2X HEPES-buffered saline. Micro tube was incubated 10-15 minutes in room temperature. Afterwards, the micro tube contents were added into medium above the cells monolayer and the cells were incubated at 37 °C. The medium was renewed 12-16 hours post-transfection, and the expression of GFP was confirmed by invert fluorescence microscopy.

Flow cytometry

The effects of HGV-apoptin and CAV-apoptin expression on the apoptosis of A549 and HEK-293 cells were assessed by PE annexin V/7-AAD flow cytometric method (BD Biosciences, USA). First, the cells were washed twice with PBS 96 hours post-transfection, and resuspended with 1X binding buffer solution. Then, 100 µl of the solution was transferred into separated collection tubes. Annexin V and 7-AAD reagents were added to each collection tube and incubated for 15 minutes at room temperature. Finally, the apoptosis was measured by FACSCalibur flow cytometry system and further analyzed by FlowJo® LLC software.

Statistical analysis

Statistical analysis of results obtained from at least three independent experiments was performed using SPSS software (Version 15). A paired Student t-test was used to calculate significance.

Results and Discussion

Our results showed that HGV-Apoptin similar to its homologue, CAV-Apoptin, induces apoptosis in A549 cells (a non-small lung carcinoma cell line), but not in

HEK-293 cells (a non-cancerous human embryonic kidney cell line). To quantify the apoptotic effects of HGV-Apoptin and CAV-Apoptin, we transfected A549 and HEK-293 cells by calcium-phosphate precipitation with pHGV-Apoptin-GFP, pCAV-Apoptin-GFP or pEGFP-C1 control vector. The GFP expression in the cells was inspected by inverted fluorescence microscopy, which indicated significant transfection efficiency (Figure 2).

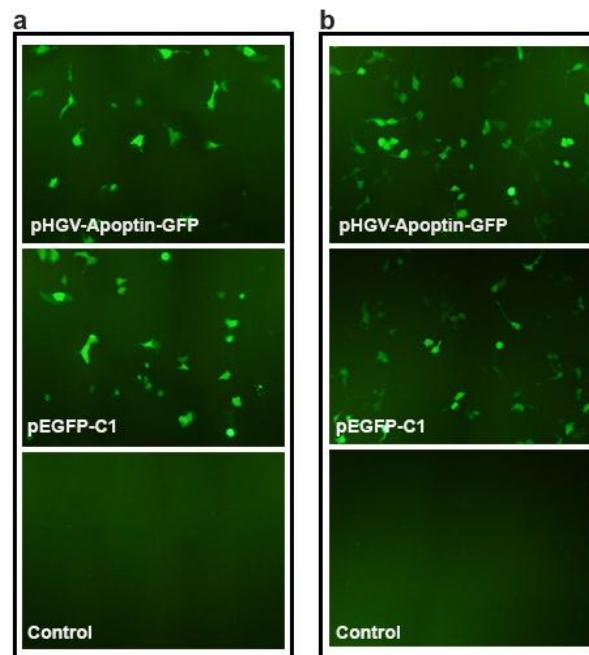


Figure 2. Transfection efficiency GFP expression and in A549 (a) and HEK-293 (b) cells inspected by invert fluorescence microscopy. Cells were imaged directly on culture plates on day four post-transfection. GFP was observed in cells cultured in the plates that were transfected with pHGV-Apoptin-GFP, pCAV-Apoptin-GFP or pEGFP-C1 expression vector (the image related to pCAV-Apoptin-GFP have not shown but it has the same efficiency as we observed for other GFP expressing plasmids). Control plates were not transfected with any vectors.

The apoptotic cells were quantified by flow cytometric analysis as a percentage of Annexin-V-positive cells among the GFP expressing cells after Annexin V-APC/7-AAD staining. In the case of A549 cell line, about 45% of the cells received HGV-Apoptin or CAV-Apoptin expressing plasmids were apoptotic compared to only about 10% apoptotic cells in those transfected with pEGFP-C1. The difference between HGV-Apoptin and CAV-Apoptin induced apoptosis in A549 cells was insignificant and both had similar apoptosis level. Although apoptosis level can be affected by various factors, the A549 apoptosis level in this study was almost similar to cancerous cells in previous studies. The ratio of cells undergoing apoptosis was less than 3% in the HEK-293 cells transfected with either pHGV-Apoptin-GFP, pCAV-Apoptin-GFP or pEGFP-C1, which were close to the obtained level of apoptosis in non-cancerous cells in previous studies.

One of the most prominent features of CAV-Apoptin is its specific cytotoxicity in cancerous cells, as shown in many studies to date.²²⁻²⁴ Despite extensive studies on CAV-Apoptin, HGV-Apoptin was discovered recently and its apoptotic effects has been evaluated on a few cell lines. In this study, we moved a step forward to show that HGV-Apoptin has cytotoxic effects on Non-small-cell lung carcinoma cell line A549, while it had no effects on non-cancerous human embryonic kidney cell line HEK-293.

Studies conducted in the past two decades, have concentrated on clarification of the functional mechanisms of CAV-Apoptin.^{9,12,25-27} Recent studies indicated that CAV-Apoptin and HGV-Apoptin have similar mechanisms and they induce apoptosis in human tumor cells without the involvement of p53 via the intrinsic mitochondrial pathway.^{19,20} It has been shown that the subcellular distribution pattern of HGV-Apoptin is the same as that observed in CAV-Apoptin, which localizes in the nuclei of cancer cells where it shows a granular distribution that later clusters to form aggregates,

while it remains in the cytoplasm of normal human cells.¹⁸ CAV-Apoptin is phosphorylated in threonine 108 mainly in tumor cells, but not in normal cells, which seems to be important in tumor-selective activation process. For a better understanding of HGV-Apoptin mechanism, it is imperative to investigate the phosphorylation patterns of this protein in normal and tumor cells.

CAV-Apoptin has been used in many cancer therapy studies due to the unique attribute in selective induction of apoptosis in cancer cells.²⁸⁻³⁰ According to our finding and previous works, HGV-Apoptin can also be used as a selective anticancer agent in cancer therapy studies. While various vectors can be used to deliver Apoptin to tumor cells, its effective delivery *in vivo* remains a challenge. Induction of apoptosis has been observed to be dependent on the intracellular level of apoptin,³¹ hence, the ideal vector should provide either a high expression of Apoptin in the tumor cells, or efficient delivery of active Apoptin protein into the cells for *in vivo* treatments.

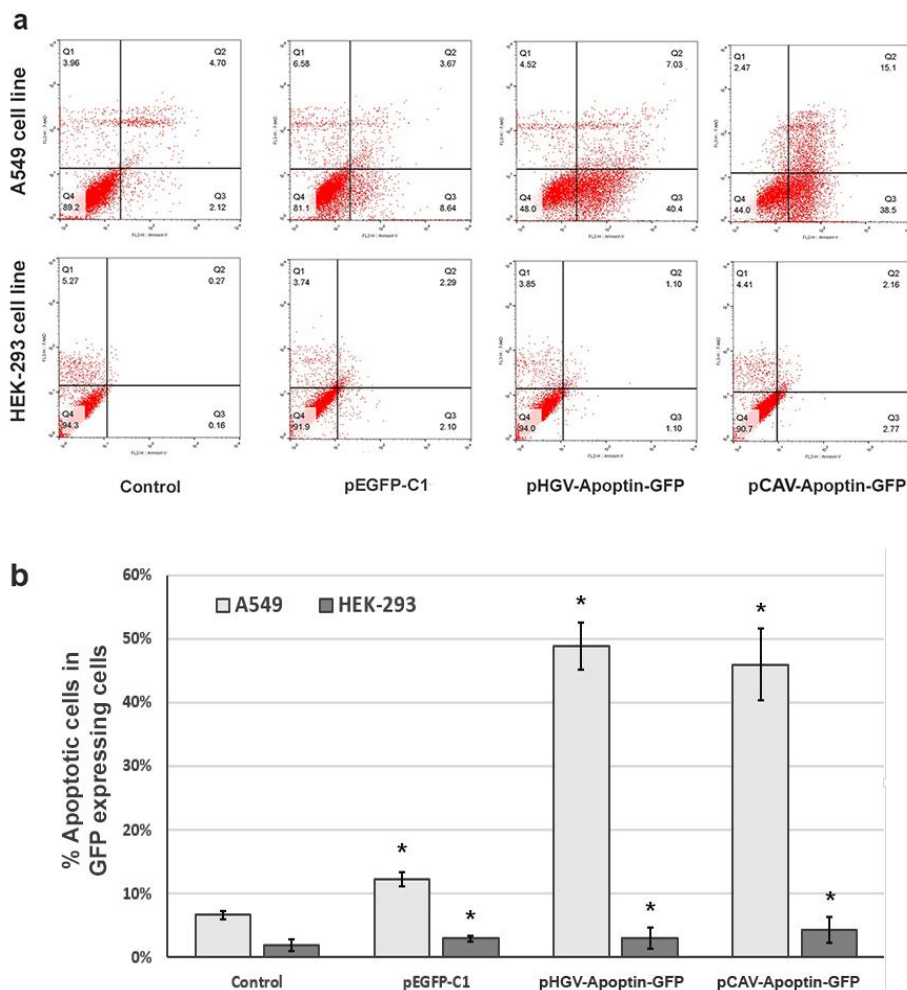


Figure 3. Quantification of apoptosis by flow cytometry after the 4th day post-transfection. A549 and HEK-293 cells were transfected with pHGV-Apoptin-GFP, pCAV-Apoptin-GFP or pEGFPC1 control vector by calcium-phosphate precipitation. **(a)** The FACS dot plots of Annexin-V-APC (x-axis) versus 7AAD (y-axis) were obtained after 4 days. A549 cell line was susceptible to CAV-apoptin and HGV-apoptin-induced apoptosis, while apoptosis level was insignificant in the control group treated with pEGFP-C1 vector and untreated groups. No apoptosis was observed in any groups of HEK-293 cell line. **(b)** Quantification of cell death as a percentage of Annexin-V-positive cells among the GFP positive cells. Error bars indicate standard deviation of three independent experiments. SPSS software was used to measure significant data. * P < 0.001.

Conclusion

In summary, our findings indicated that HGV-Apoptin and CAV-Apoptin had cytotoxic effects on Non-small-cell lung carcinoma cell line A549 while these proteins had no effects on non-cancerous human embryonic kidney cell line HEK-293.

According to our result as well as previous studies, HGV-Apoptin similar to its homologue, chicken anemia virus derived Apoptin (CAV-Apoptin) can be introduced as a potential gene oncotherapeutic agent. Further investigation can provide more insight regarding this issue.

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Conflict of interests

The authors claim that there is no conflict of interest.

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