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Original Research

Oncolytic E1B 55KDa-deleted adenovirus replication is independent of p53 levels in cancer cells

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Abstract: Oncolytic adenoviruses represent a new approach for cancer therapy due to its tumor specificity. E1B 55kDa-deleted adenovirus type 5 (Ad5dlE1B 55kDa) is a promising therapeutic agent that can selectively replicate in and lyse p53 defective cancer cells. However, the overall efficacy has shown varying degrees of success with raised doubts about the correlation between p53 status and E1B-deleted adenovirus replication ability. In this study, we investigated the relationship between the efficiency of Ad5dlE1B 55kDa replication and p53 levels in cancer cells. Five transient p53 expression vectors were engineered to expresses different p53 levels in transfected cells. Then, the effect of the variable p53 levels and cellular backgrounds on the replication efficiency of oncolytic Ad5dlE1B 55kDa was evaluated in H1299 and HeLa cell lines. We found that the replication efficiency of these oncolytic viruses is dependent on the status, but not the expression levels, of p53. Ad5dlE1B 55kDa was shown to have selective replication activity in H1299 cells (p53-null) and decreased viral replication in HeLa cells (p53-positive), relative to the wild-type adenovirus in both cell lines. Our findings suggest that there is a relation between the E1B-deleted adenovirus replication and the presence as well as the activity of p53, independent of its quantity.

Key words: Oncolytic adenovirus; p53; E1B 55kDa; H1299 cells; HeLa cell.

Introduction

Recombinant Adenoviruses are widely used as therapeutic vectors in cancer treatment. Recently, a new promising concept has been developed based on using replication-competent mutant adenovirus known as an oncolytic adenovirus. To date, there are over 1500 cancer gene therapy clinical trials, where oncolytic viruses represent more than half of the trials (1). Oncolytic viruses have the capability to replicate selectively in and destroy cancer cells (2, 3). In most cancers, the function of p53 tumor suppressor gene that controls cancer progression and maintains DNA integrity is lost, resulting in the defective p53 pathway (4, 5). The latter causes uncontrolled cellular checkpoints and failure in the induction of apoptosis (6). Fascinatingly, adenovirus exploits for its replication the same altered pathways in cancer, as it is found to target and inactivate p53(7).

Adenovirus E1B 55kDa protein can directly interact with tumor suppressor p53 protein through a sequence-specific mechanism (8-10). This interaction results in loss of p53 function to suppress the ability of the virus to transform cells, replicate viral DNA and establish a lytic infection (10, 11). The suppression is mediated by the transcription of a large set of p53 target genes, which are involved in many biological outcomes as a result of p53 tumor suppressor activity including cell cycle arrest, DNA repair, microRNA processing and apoptosis (12-16). Thus, E1B 55kDa viral gene becomes dispensable in cancer cells that have lost the target protein. This gene is the primary target of modification for creating tumor selective replicative adenoviruses (17). Based on these facts, the first tumor-restricted adenovirus (*dl1520)* was engineered and tested *in vitro* by Barker and Berk (18). The basis for tumor selectivity has been postulated to be a deletion of 827bp in the viral E1B 55kDa gene, which would result in selective replication of E1B 55kDa-deleted adenovirus in p53-deficient tumor cells, as E1B 55kDa viral gene becomes superfluous (19).

Similarly, Frank McCormick and his group found that *dl1520* replication *in vitro* was more restricted to p53-deficient tumor cells (20). Besides, the same group was the first to propose the notion of treating tumors lacking p53 function with an EIB-deleted adenovirus (21). As a result of its efficient reduction of tumor size, E1B-deleted adenovirus was approved as the first successful adenovirus mutant for clinical use under the name of ONYX-015. Accordingly, a series of clinical trials in treating 250 participants of different cancer types have been initiated (22). ONYX-015 has shown success in the early stage with low toxicity rate and well tolerance (23). However, the available data from phase I and phase II of head and neck clinical trials have shown the limited efficacy of E1B-deleted adenovirus, as 50% of the cases has shown clinical benefits in combination with chemotherapy (24). Moreover, ONYX-015 as a single agent exhibited a low degree of efficacy with 13-14% complete tumor regression in another clinical set

(25).

The mechanism of selectivity of this oncolytic virus is more complicated than what was initially assumed, as it does not depend on hindering viral RNA transportation or hindering virus spread by neutralizing antibodies, but rather depends on the difference in p53 status (26, 27). Previous studies performed on RKO and H1299 cancer cell lines reported a significant reduction in virus replication efficiency in both cell lines with positive p53 compared to the matched cell lines with negative p53 (28, 29). On the other hand, another study was done by Shen and co-workers showed the unclear inconsistency of the replication of multiple mutant E1B adenoviruses in variable p53 levels that were detected in U2OS cells infected with different ONYX as well as adenovirus dl309 (30). The lack of real correlation between p53 and E1B 55kDa-deleted adenovirus replication ability was assumed to be the reason of such contradictions (31). In this study, we examined the replication efficiency of E1B 55KDa-deleted adenovirus (Ad5dlE1B 55kDa) in cancer cell lines that have different p53 cellular backgrounds and under varying levels of plasmid-encoded p53 to determine the role of p53 in modulating E1B 55kDa-deleted adenovirus replication.

Materials and Methods

Plasmid construction

Six plasmids were engineered by cloning the p53 gene and five regulatory elements in an additive manner into the pUC19 backbone. The regulatory elements were used to achieve varying levels of transiently expressed p53, by increasing the stability of either plasmids DNA or p53 mRNA in mammalian cells. Polymerase chain reaction (PCR) was used to obtain the p53 promoter that was cloned between *Zra*I *and Nde*I sites of pUC19 to generate plasmid pUC19-p53 promoter. The p53 expressing plasmid (pA) was then constructed by subcloning of the p53 gene obtained by reverse transcription of human RNA followed by PCR. The obtained p53 gene fragment was cloned into the *Nde*I/*EcoR*I sites of plasmid pUC19-p53 promoter. PCR product containing matrix attachment region I (MARI) was also cloned upstream of p53 in plasmid pA to generate pB. Another PCR product containing MARII was then cloned downstream of p53 to generate plasmid pC. The HPRE-containing plasmid (pD) was constructed by cloning the human posttranscriptional regulatory element (HPRE) at the *Bam*II site of plasmid pC. Finally, plasmid pE was engineered by annealing two synthesized oligonucleotides (Integrated DNA Technology, IDT) to construct the bovine growth hormone (BGH) poly-A signal followed by cloning downstream of the HPRE element into the *Bam*II site of plasmid pD. T4 DNA ligase (New England Biolabs) was used to clone the different fragments into the target plasmid according to the manufacturer's instructions. The backbone pUC19 plasmid was used as a negative control and was designated pF. The different engineered plasmids are shown in Table 1 and oligos and primers sequences are listed in Table 2.

Plasmid DNA preparation

Small-scale and large-scale plasmid DNA isolations were carried out using Plasmid DNA Miniprep Kit and Plasmid DNA Maxiprep Kit (Norgen Biotek) according to the manufacturer's instructions. Purified plasmids were then stored at -20°C for further use.

Cell lines

H1299 Human non-small-cell lung carcinoma (American Type Culture Collection (ATCC) CRL-5803) and HeLa cells (ATCC CCL-2) (32) were used in this study. H1299 cells were maintained and cultured as a monolayer in RPMI-1640 Medium (Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS, Innovative Research) and 1% (v/v) penicillin/streptomycin (Gibco). In contrast, HeLa cells were maintained as a monolayers in Dulbecco's Minimum Essential Medium (DMEM, Gibco), supplemented with 5% (v/v) fetal bovine serum (FBS, Innovative Research) and 1% (v/v) penicillin/streptomycin (Gibco). Both cells were passaged twice weekly until they reached 90% confluencey using Ethylenediaminetetraacetic acid-trypsin (EDTA-trypsin) and 0.05% phenol red solution (Gibco), according to ATCC's instructions. In brief, the medium was aspired and cells were washed with 6 mL of phosphate buffered saline (PBS) pH 7.4 (Gibco). Two milliliters of EDTA-Trypsin were then added to the cell monolayer in a 150 mm plate. After 2-3 minutes, cells were lifted by gently tapping the plate's sides and 4-6 mL of the complete growth medium were added to stop the trypsin effect. A new culture plate was then filled with the medium and 2 mL of the cell suspension were added and cultures were incubated in a water-jacketed incubator (Fisher Scientific) at 37°C with 96% relative humidity and 5% CO_2.

Viruses

Three viruses were used in this study; EIB55kDa mutant adenovirus (Ad5dlE1B 55kDa) that has a dele-

Table 1. List of adenoviruses and plasmids used in this study.

Name	Description
Ad5dlE1B 55KDa	Adenovirus with deleted E1B 55kDa and E3
AdGFP	Adenovirus with deleted E1 as well as E3 and contains GFP gene
Ad5wt	Adenovirus with deleted E3
pA	p53 promotor + p53 gene
pB	p53 promotor + p53 gene + MARI
pC	MARII + p53 promotor + p53 gene + MARI
pD	MARII + p53 promotor + p53 gene + HPRE + MARI
pE	MARII + p53 promotor + p53 gene + HPRE + polyA + MARI
pF	Negative control (pUC19)

Table 2. List of primers, probes and oligonucleotides.

Gene or fragment	Sequence	Annealing temperature (°C)	Amplicon size (bp)
P53 promoter	5'CTAGATGACGTCCCTAGGAGATCTCGAGGGGAGAAAACGTTAGGGTGTG3' 5'GTCGAGCATATGGGCAGTGACCCGGAAGGCAG3'	60	532
P53 gene	5'CTAGATCATATGGTCTAGAGCCACCGTCCAGG3' 5'CTAGATGGCGCCTCAGTCTGAGTCAGGCCCTTC3'	54	1317
P53 Sequencing Primers	5'TCCCTTCCCAGAAAACCTACC3' 5'GGCGCCTCAGTCTGAGTCAGGCCCTTC3'		
P53 TaqMan	5'TAACAGTTCCTGCATGGGCGGC3' 5'AGGACAGGCACAAACACGCACC3' 5'/FAM/CGGAGGCCC/ZEN/ATCCTCACCATCATCA/3IABkFQ/3'	59	121
MARI	5'TCCCCGGGTTAGTAAGACATCACCTTGCATTT3' 5'AACAGCCATAGTTTGAGTTACCCTTT3'	50	770
MARII	5'TGCCTAGGTTAGTAAGACATCACCTTGCATTT3' 5'TGCTCGAGAGCCATAGTTTGAGTTACCCTTT3'	50	770
HPRE	5'CTAGATGGCGCCTTGCTCGGCAACGGCC3' 5'CTGAATTCGACATTGCTGGGAGTCCAAGAG3'	54	534
BGH poly-A	5'TCGAATTCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCG-TGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAA-TGAGGAAATTGCAT3' 5'TCCCCGGGGCCTGCTATTGTCTTCCCAATCCTCCCCCTTGCTGTCCTGCCC-CACCCCACCCCCCAGAATAGAATGACACCTACTCAGACAATGCGATG-CAATTTCCTCATTTTATTAGGA3'	68	198
BGH poly-A Sequencing primers	5'TCGAATTCTGTGCCTTCTAGTTGCCAGC3' 5'ACCCGGGGGCCTGCTATTGTCTTCCCA3'		
Adenovirus hexon	5'CGGGTAATATGGGTGTTCTGG3' 5'GCTCTGTGTTTCTGTCTTGC3' 5'/FAM/CATTCAACT/ZEN/GCGATGCTTGGCCC/IABkFQ/3'	50	78
GAPDH TaqMan	5'AAATTCAACCTCTTGGGCCCTCCT3' 5'AGGCGCCCAATACGACCAAATCTA3' 5'/FAM/AGGAGATGC/ZEN/TGCATTCGCCCTCTTAA/3IABkFQ/3'	56	156
S15 TaqMan	5'CTACAACGGCAAGACCTTCA3' 5'GGCTTGTAGGTGATGGAGAAC3' 5'/FAM/AGGTGGAGA/ZEN/TCAAGCCCGAGATGA/3IABkFQ/3'	51	182
5S TaqMan	5'GCCATACCACCCTGAACCG3' 5'AGCCTACAGCACCCGGTATT3' 5'/FAM/TTAGTACTT/ZEN/GGATGGGAGACCGCCT/3IABkFQ/3'	54	111

*Underlined sequence indicates extra sequence containing restriction sites.

tion of 827bp in the EIB55kDa protein-coding region and mutant adenovirus with a deletion in the E1 region and with GFP reporter gene (AdGFP), as well as the phenotypically wild-type adenovirus type 5 (Ad5wt) that lacks the entire E3 region. These viruses were available in our laboratory from previous studies (33, 34). All viruses were propagated in HEK 293 cells which are transformed with the adenovirus E1 gene, making it a permissive cell line for adenovirus production (35). Briefly, 2mL of viral inoculum were diluted in 8mL of PBS^{++} infection medium (0.01% $CaCl_2.2H_2O$ and 0.01% $MgCl_2.6H_2O$ dissolved in PBS). At 90% confluency, the culture medium was aspirated and the infection medium was added to the cells and incubated at 37°C for an hour while rocking the plate. Following incubation, 20 mL of growth medium were added and incubated at 37°C overnight. Cells were monitored for the cytopathic effect (CPE) for days and when they displayed complete CPE, they were removed with a cell scraper (BD Falcon). Cells and medium were then transferred to a 50 cc tube and centrifuged at 1000xg for 5 minutes. The supernatant was supplemented with 10% sterile glycerol and stored at -80°C, whereas cell pellet underwent several freeze and thaw cycles to release progeny virions. Viral yields were then subjected to titer determination using quantitative PCR (qPCR) assay and end-point titration assay (36, 37).

Plasmid transfection

To establish cell lines expressing varying levels of p53 for a prolonged period of time, an *in vitro* transfection of different molar ratio from each plasmid (pA, pB, pC, pD and pE) in addition to pUC19 (pF), the negative control, was conducted using Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer's protocols. The experiment was repeated three times and triplicate samples were used in each experiment.

Viral infection and replication analysis

Transfected cell lines were further infected with Ad5wt, Ad5dlE1B 55kDa or AdGFP at 24 hours post-transfection. The infected pUC19-transfected cells were used as negative control. Monolayers of the indicated cell lines were infected with 100 µL/well (10 PFU per cell) and incubated for an hour at 37°C in an incubator

using 5% CO_2 and 96% humidity, with swirling every 15 minutes. Two milliliters of normal growth medium were then added back to each well and the plate was maintained in the incubator under the same conditions. Each sample was done in triplicate and the experiment was repeated three times. Samples were collected at 2, 24, 48 and 72 hours post-infection. Total DNA and total RNA were isolated using RNA/DNA/Protein Purification Kit (Norgen Biotek). On column DNase digestion step was used during the RNA isolation to eliminate any DNA carryover. Isolated DNA and RNA samples were then quantified using NanoDrop 2000 Spectrophotometer (Thermo Scientific). Samples with 260/280 ratio value between 1.8 and 2.0 were considered pure. TaqMan-based qPCR reactions were then performed on the CFX Connect Real-time PCR Detection System (Bio-Rad) to assess viral DNA replication efficiency by measuring viral DNA levels per cell. Specific probes and primers for the adenoviral hexon gene and two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 5S rRNA, were utilized to amplify the desired region (Table 2). GAPDH and S5 were used with H1299 and HeLa cells, respectively, as they are the specific highly stable genes in each cell line (38-41). The reaction mixture contained 3 µL of DNA template, 10 µL of 2X PCR Master Mix (Norgen Biotek Corp.), 2 µL of primer mix (5 µM, each), 1 µL of specific probe (10 µM) of each gene in a final reaction volume of 20 µL. The TaqMan qPCR program was performed as follows: initial incubation at 95°C for 3 minutes followed 40 cycles of denaturation for 15 seconds at 95°C then annealing and extension for 30 seconds at 60°C. Viral DNA input was used to normalize the measurement of viral DNA level. The calculation of relative viral DNA levels per cell was done using ΔCT threshold method by either of the following two equations: $2^{\Delta(Ct\ GAPDH\ -\ Ct\ hexon)}$ or $2^{\Delta(Ct\ 5S\ -\ Ct\ hexon)}$.

Reverse transcription

Complementary DNA (cDNA) was prepared from purified RNA through reverse transcription (RT) using the Superscript III system (Invitrogen). In the initial denaturation step, 3 µL RNA were mixed with 0.5 µL of 100 mM oligo(dT) 18-mer primer (IDT) and DNase/RNase free water in a reaction volume of 10 µL. The reaction was incubated at 70°C for 5 minutes followed by cooling at 4°C for 5 minutes. During the cooling step, 10 µL mastermix was added to each sample. The mastermix consists of 4 µL of the 5X First Strand Buffer, 2 µL of 0.1 M Dithiothreitol, 1 µL of 10 mM dNTPs, 0.1 µL of Superscript III (SSIII, Invitrogen), and 2.9 µL of DNase/RNase free water. The reaction was then incubated at 25°C for 5 minutes, followed by 42°C for 60 minutes and a final incubation at 70°C for 15 minutes before holding the reaction at 4°C.

P53 expression analysis

P53 expression levels as well as the human ribosomal protein S15 levels were measured in Ad5wt-, Ad-5dlE1B 55kDa- or AdGFP-infected H1299 and HeLa cells by using TaqMan-based qPCR assays. In brief, the isolated and quantified RNA samples were analyzed for their quality using the Agilent RNA 6000 kit with the Agilent 2100 Bioanalyzer (Agilent Technologies Inc.). Reverse transcription followed by qPCR assay were then conducted on the RNA samples to assess the p53 gene expression levels per cell. The reaction was performed using specific probe and primers (Table 2) and the same TaqMan qPCR program used earlier to analyze viral replication. Cells transfected with pUC19 (pF) and no template control transfection were used as negative controls, however only pUC19 transfected cells were used to normalize the measurement of p53 expression level. Relative expression levels of p53 per cell were measured by the ΔCT threshold method according to the following equation: $2^{\Delta(Ct\ S15\ -\ Ct\ p53)}$.

Cytopathic effect (CPE) assay

H1299 and HeLa cells were infected with 10 PFU/cell of Ad5dlE1B 55KDa, AdGFP or Ad5wt at 24 hours post-transfection. Plates were then incubated at 37°C and monitored daily. Viral CPE of the different adenoviruses was determined morphologically over 10 days post-infection using an inverted microscope. Samples were prepared in triplicate and three independent experiments were carried out. Uninfected wells were used as a control.

DNA sequencing

DNA sequencing in all of the cloning steps was carried out to verify sequence accuracy of the PCR product as well as the final construct. Sequencing was performed on the Applied Biosystem 3130x Genetic Bioanalayzer DNA sequencer (Applied Biosystems). Cycle sequencing reaction was carried out along with BigDye Terminator chemistry using the BigDye Terminator v1.1 Sequencing Standard Kit (Applied Biosystems). Primers used in the initial PCR to obtain each cloned elements were used as sequencing primers, with the exception of the p53 gene and the BGH poly-A fragment, where specific sequencing primers were used (Table 2). Sequencing data were analyzed using Finch TV software as a base calling program.

Statistical analysis

All statistical analysis was performed using Excel (Microsoft). The analysis of variance one-way ANOVA and the Student's t-test were utilized in this study to calculate the significance of viral DNA levels and p53 expression levels per cell. P values of <0.05 were considered significant.

Results

The replication efficiency of Ad5dlE1B 55kDa was evaluated in both HeLa cells and H1299 cells since they have different p53 backgrounds. HeLa cells express endogenous wild-type functionally inhibited p53 while H1299 cells are p53 deficient. Both cell lines were transfected with five p53 expression vectors (pA to pE), which express the different amount of exogenous p53 (data not shown), in addition to a negative control plasmid pUC19 (pF). As a result, six different p53 backgrounds were obtained for each cell line. Three different viruses were investigated for their replication efficiency upon infecting these cellular conditions. The first virus is Ad5dlEIB 55kDa (Ad5dlE1B 55KDa), an Ad with deleted E1B 55kDa region (827bp) and the entire E3 re-

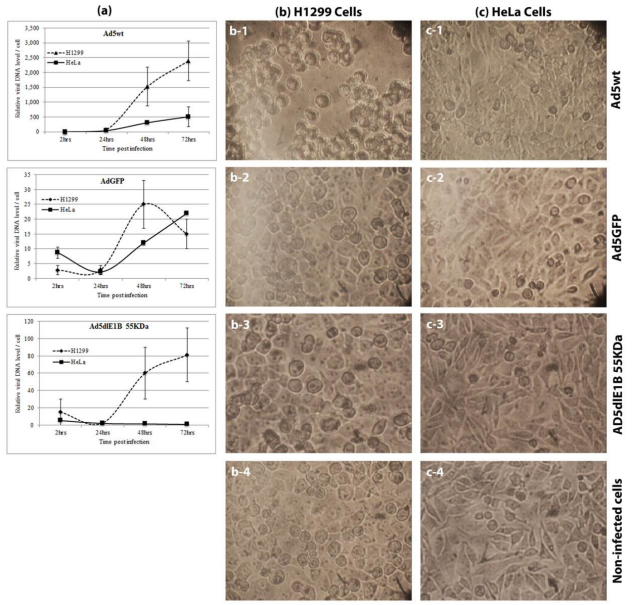

Figure 1. (a) Growth rate of Ad5wt, AdGFP and Ad5dlE1B 55KDa in H1299 cells and HeLa cells. Relative viral DNA levels over 2, 24, 48 and 72 hours post-infection were measured after normalization with cellular gene using the equation $2^{\Delta(Ct\ cellular\ gene\ -\ Ct\ hexon)}$. Error bars represent the standard deviation. Cellular morphology of (b) H1299 cells and (c) HeLa cells at 72 hours post-infection with Ad5wt, AdGFP and Ad5dlE1B 55kDa at MOI of 10 PFU/cell. The control represents non-infected cells. Pictures were processed using the MotiCam 480 digital camera (Motic Instruments) using an inverted microscope at x200 magnification.

gion. This virus lacks the ability to interact with p53 but still contains E1B 19kDa that prevents premature death of host cell through apoptosis and to achieve transformation. The second virus is E1-deleted Ad (AdGFP) which encodes GFP reporter gene and is replication-deficient due to the deleted entire E1 and E3 regions. The third virus is the wild-type Ad (Ad5wt) with complete deletion of the E3 region. Since the E3 region expresses the death protein, it was entirely deleted in all viruses to avoid the early death of the infected cell and to facilitate viral replication. Both AdGFP and Ad5wt viruses were used in this study as viral controls.

Characterization of viral replication in H1299 and Hela cells

The replication phenotype of Ad5wt, AdGFP, and Ad5dlE1B 55kDa in H1299 and HeLa cell lines was characterized to evaluate the role of the cellular p53 in viral replication efficiency. Viral infection with Ad5dlE1B 55KDa, AdGFP, and Ad5wt was carried out at MOI of 10 PFU/cell, which was optimized in another experiment (data not shown). Viral DNA synthesis was used as an indicator of replication efficiency. The latter was determined by measuring relative viral DNA levels per cell using the Delta Ct (ΔCT) threshold method. The results showed that replication efficiency of Ad5wt and Ad5dlE1B 55kDa was significantly higher in H1299 cells compared to HeLa cells at all the assessed time points post-infection (Figure 1-a, p-value >0.05). The replication efficiency of AdGFP was significantly higher in H1299 cells only at 48 hours post-infection.

The CPE of the three viruses was directly correlated with the viral genome replication in both cell lines. Ad5wt killed more than 50% of the cells by day three in both cell lines revealing the efficiency of viral production (Figure 1-b and 1-c). In contrast, cells infected with Ad5dlE1B 55kDa did not show visible CPE in Hela cells and was slower to produce CPE in H1299 cells compared to Ad5wt. Ad5dlE1B 55kDa induced complete lysis of H1299 cells by day ten post-infection (data not shown). On the other hand, both H1299 and HeLa cell lines were resistant to lysis by AdGFP.

Effect of p53 levels on viral replication efficiency in H1299 cells

We examined the effect of p53 levels on the efficiency of viral replication in H1299 cells. First, we prepared panels of 6 conditions of H1299 cells transfected with the different p53 expression vectors (pA to pE) that express varying amounts of the p53 and pUC19 vector (pF) that does not encode p53 transgene (negative control). Then we infected the transfected cells with the adenovirus vectors (one virus to infect each panel). DNA and RNA samples were isolated, cDNA was prepared from the RNA samples using reverse transcription and both DNA and cDNA were used in probe based-TaqMan qPCR to assess the relative viral DNA levels and relative p53 expression level per cell using the ΔCT calculation method.

In Ad5wt-infected H1299 cells, the virus replicated efficiently in presence or absence of wild-type expressed p53 (pA to pF). Cells that are transfected with plasmid pF (null p53 cells) have insignificantly higher levels of viral DNA compared to cells transfected with p53 expression vectors (p value= 0.837) (Figure 2). This indicates that the different levels of p53 did not signifi-

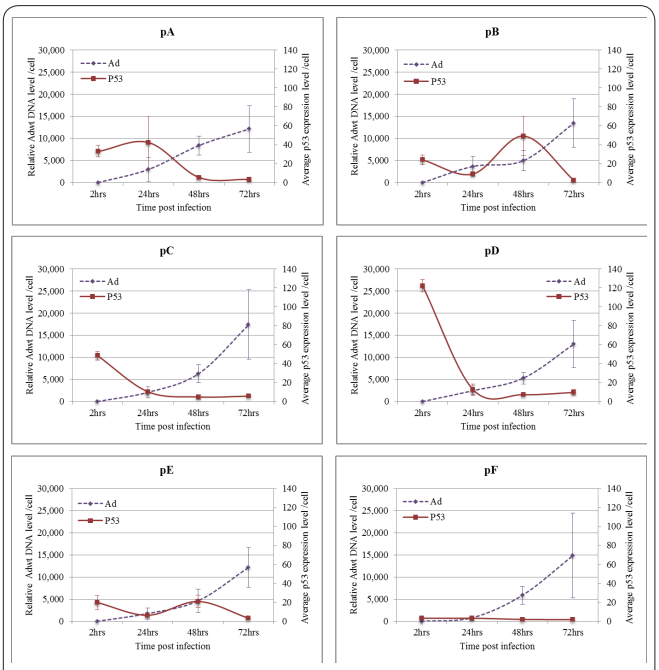

Figure 2. Effect of p53 levels on Ad5wt replication efficiency in H1299 cells. Relative viral DNA levels were assessed over 2, 24, 48 and 72 hours post-infection, under the different p53 levels from plasmids pA to pE. Plasmid pF is the negative control. Relative DNA levels were normalized to GAPDH gene levels using the equation $2^{\Delta(Ct\ GAPDH\ -\ Ct\ hexon)}$, while relative p53 levels were normalized using the S15 gene expression and were calculated using the equation $2^{\Delta(Ct\ S15\ -\ Ct\ p53)}$. Error bars represent the standard deviation.

cantly affect the replication level of Ad5wt. However, in AdGFP –infected H1299 cells, an opposite pattern was observed, where AdGFP did not replicate efficiently in all conditions (with or without respect to wild-type p53). The different p53 levels that are expressed from the various plasmids (pA to pE) have an insignificant effect on the efficiency of viral replication. Viral DNA levels reached its highest in cells transfected with plasmids pA and pB at 72 hours post-infection. However, this increase in the replication capacity was insignificant when compared to viral replication in the other plasmids' conditions (p-value >0.05). These results confirm the above finding in which H1299 cells are restrictive for AdGFP, which showed an impaired replication in the presence or absence of p53. A very low p53 amount was observed at 24 hours post-infection from plasmid F for unknown reason, as this plasmid does not encode a p53 transgene (Figure 3).

On the other hand, the efficiency of Ad5dlE1B 55kDa replication was in direct correlation with the presence of functionally active p53. A weak viral replication was observed in cellular conditions transfected with p53 expression plasmids pA to pE. However, the various levels of p53 in infected H1299 cells did not significantly affect the level of replication of Ad5dlE1B 55kDa. In H1299 cells transfected with the negative control plasmid pF, DNA levels of Ad5dlE1B 55kDa started to increase earlier than the other conditions at 24 hours post-infection and continued to rise to be the highest at 72 hours post-infection, when compared to plasmids pA to pE (p-value <0.05) (Figure 4).

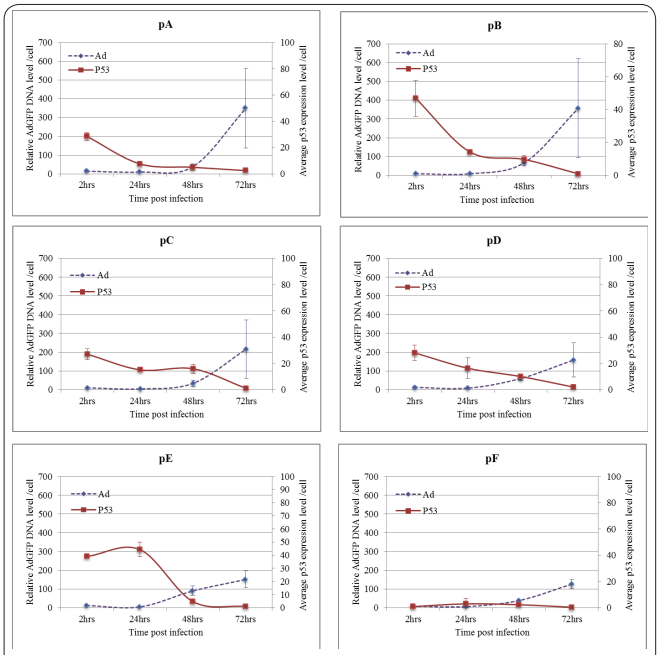

Figure 3. Effect of p53 levels on AdGFP replication efficiency in H1299 cells. Relative viral DNA levels were measured over 2, 24, 48 and 72 hours post-infection, under the different p53 levels from plasmids pA to pE. Plasmid pF is the negative control. Relative DNA levels were normalized to GAPDH gene levels using the equation $2^{\Delta(Ct\ GAPDH\ -\ Ct\ hexon)}$, while relative p53 levels were normalized using the S15 gene expression and were calculated using the equation $2^{\Delta(Ct\ S15\ -\ Ct\ p53)}$. Error bars represent the standard deviation.

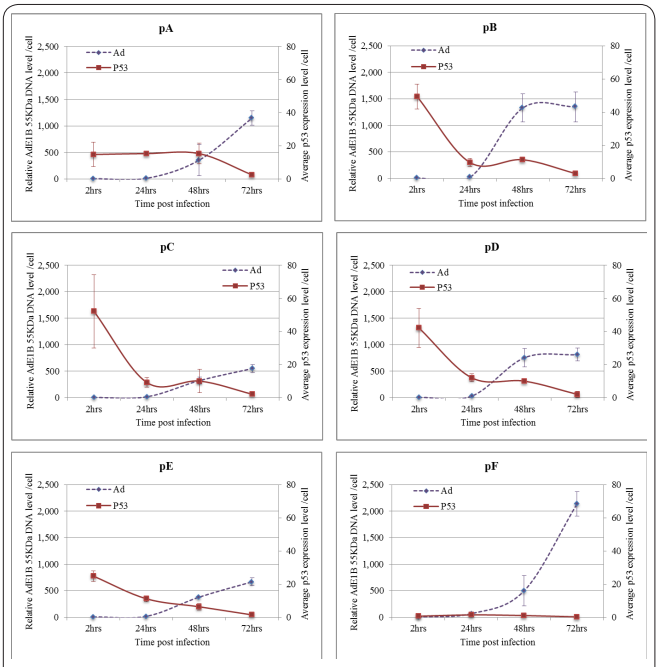

Figure 4. Effect of p53 levels on Ad5dlE1B 55KDa replication efficiency in H1299 cells. Relative viral DNA levels were measured over 2, 24, 48 and 72 hours post-infection, under the different p53 levels from plasmids pA to pE. Plasmid pF is the negative control. Relative DNA levels were normalized to GAPDH gene levels using the equation $2^{\Delta(Ct\ GAPDH\ -\ Ct\ hexon)}$, while relative p53 levels were normalized using the S15 gene expression and were calculated using the equation $2^{\Delta(Ct\ S15\ -\ Ct\ p53)}$. Error bars represent the standard deviation.

Effect of p53 levels on viral replication efficiency in HeLa cells

To further confirm that Ad5dlE1B 55kDa replication is not dependent on p53 levels, but rather to its functional activity, we performed the same experiments in HeLa cells. Viral replication efficiency of Ad5wt, AdGFP, and Ad5dlE1B 55kDa was determined under different p53 backgrounds, including HeLa cells expressing its endogenous p53 and HeLa cells expressing both endogenous and exogenous p53 from plasmids pA to pE. Quantitative PCR followed by ΔCt threshold method were performed on the DNA samples to assess relative viral DNA levels per cell. Similarly, relative p53 expression levels were also determined using reverse transcription on RNAs from the same sample followed by qPCR.

Ad5wt replicated efficiently in the presence or absence of p53 in transfected-HeLa cells, and the various p53 levels showed an insignificant effect on its replication efficiency. Viral DNA levels started to increase at different time points in all of the conditions and reached various levels at 72 hours post-infection (Figure 5). Expression levels of p53 attained its maximum peak at 2 and 24 hours post-infection and started to decrease afterward to reach the basal cellular levels. Also, insignificant differences of viral DNA levels were obtained from all conditions at 48 hours post-infection (p values >0.05).

In HeLa cells infected with AdGFP, the virus replicated efficiently in the transfected cells, with different viral DNA levels obtained from the various conditions (pA to pE). AdGFP showed the highest DNA levels in conditions pB and pE at 72 hours post-infection. Howe-

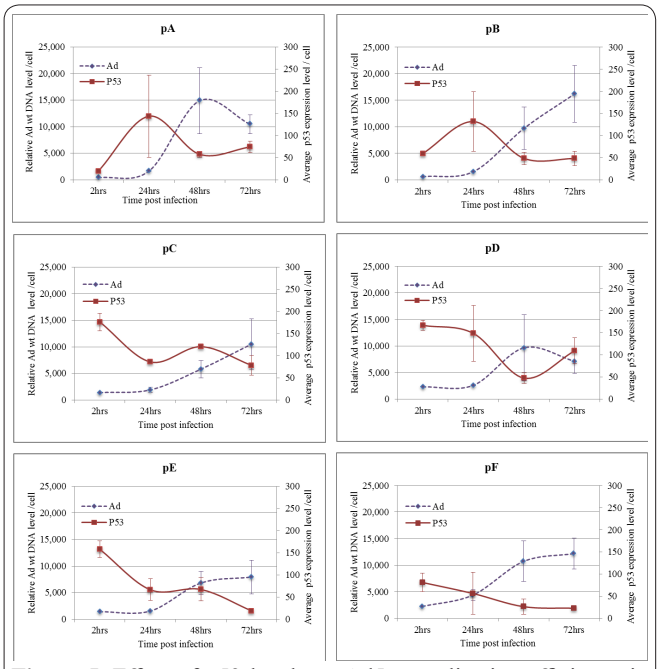

Figure 5. Effect of p53 levels on Ad5wt replication efficiency in HeLa cells. Relative viral DNA levels were measured over 2, 24, 48 and 72 hours post-infection, under the different p53 levels from plasmids pA to pE. Plasmid pF is the negative control. Relative DNA levels were normalized to 5S using the equation $2^{\Delta(Ct\ 5S - Ct\ hexon)}$, while relative p53 levels were normalized using the S15 gene expression and were calculated using the equation $2^{\Delta(Ct\ S15 - Ct\ p53)}$. Error bars represent the standard deviation.

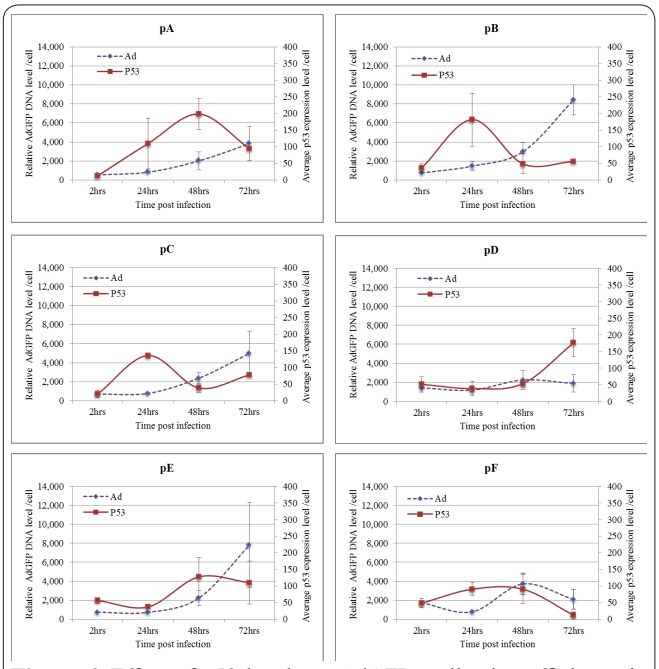

Figure 6. Effect of p53 levels on AdGFP replication efficiency in HeLa cells. Relative viral DNA levels were measured over 2, 24, 48 and 72 hours post-infection, under the different p53 levels from plasmids pA to pE. Plasmid pF is the negative control. Relative DNA levels were normalized to 5S using the equation $2^{\Delta(Ct\ 5S - Ct\ hexon)}$, while relative p53 levels were normalized using the S15 gene expression and were calculated using the equation $2^{\Delta(Ct\ S15 - Ct\ p53)}$. Error bars represent the standard deviation.

ver this increase was insignificant (p-value >0.05). Cells transfected with plasmid pB showed significantly higher viral DNA replication than the negative control cells transfected with plasmid pF (pF) (p-value <0.05), although p53 expression pattern of plasmid pB was almost the same compared to the other plasmids (Figure 6).

Ad5dlE1B 55kDa replication efficiency in HeLa cells showed significantly lower DNA levels than Ad5wt and AdGFP. As shown in Figure 7, the highest levels were at 2 hours post-infection, and it sharply reduced after this point reaching either the basal levels or zero at 72 hours post-infection, indicating an active p53, which is unlikely in this cell line. However, the inefficient replication of Ad5dlE1B 55kDa in all conditions strongly supports the above suggestion. On the other hand, no significant differences in viral DNA levels per cell were found under the varying levels of p53 (Figure 7), which reconfirmed the results that were obtained from H1299 cells.

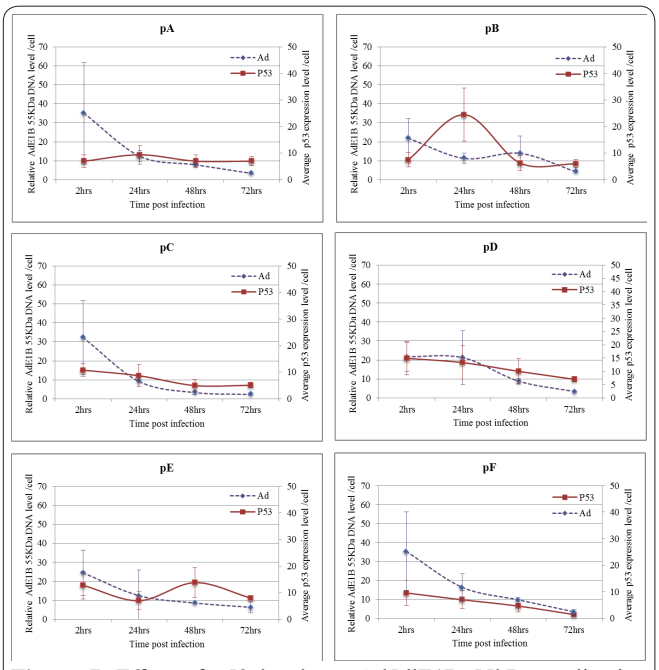

Figure 7. Effect of p53 levels on Ad5dlE1B 55kDa replication efficiency in HeLa cells. Relative viral DNA levels were assessed over 2, 24, 48 and 72 hours post-infection, under the different p53 levels from plasmids pA to pE. Plasmid pF is the negative control. Relative DNA levels were normalized to 5S using the equation $2^{\Delta(Ct\ 5S - Ct\ hexon)}$, while relative p53 levels were normalized using the S15 gene expression and were calculated using the equation $2^{\Delta(Ct\ S15 - Ct\ p53)}$. Error bars represent the standard deviation.

Discussion

Wild-type tumor suppressor p53 protein is a well-known cellular defense against DNA damage and oncogenic activity, specifically to viral infection (42). Several studies showed that oncolytic E1B 55kDa-deleted adenovirus can preferentially replicate in p53-deficient cancer cells, with mutant or null p53 status, or sometimes in cells with wild-type p53 for ambiguous reasons (24, 29, 43, 44). In this study, we evaluated the replication efficiency of E1B 55kDa-deleted adenovirus (Ad5dlE1B 55kDa) in different p53 backgrounds and levels to find out the mechanism behind its replication selectivity.

Since normal cells are not an option for adenovirus oncolysis, H1299 (p53-null) and HeLa (wild-type, functionally inhibited p53) cell lines were chosen for this study. H1299 does not contain the p53 gene or p53 ex-

pression (45), while wild-type p53 status of HeLa cells is functionally inhibited due to the binding of HPV E6 to p53, causing blockage of its transactivation function. This blockage has been reported as a result of inhibition of p53 acetylation and phosphorylation by HPV E6 region (46-48). In addition to Ad5dlE1B 55kDa, another two viruses were used; wild-type adenovirus type 5 (Ad5wt) and mutant adenovirus with a deletion in the E1 region and with GFP reporter gene (AdGFP). The growth rate of Ad5wt, AdGFP, and Ad5dlE1B 55KDa in different p53 backgrounds cell lines, H1299 and HeLa, was examined. The obtained results (Figure 1-a) indicated that H1299 cells were more supportive for Ad5wt and Ad5dlE1B 55kDa replication than HeLa cells. The high replication rate in H1299 cells was expected as they do not contain p53 expression that can hinder viral growth. Unfortunately, this advantage of H1299 cells did not facilitate AdGFP replication, as this virus is lacking the replication genes (ΔE1) that are essential for its production; therefore, its replication is hindered.

Although Ad5wt and Ad5dlE1B 55kDa seem to have a similar replication phenotype, DNA levels of Ad5wt were significantly higher when compared to Ad5dlE1B 55kDa in the H1299 cell line (Figure 1-a). This indicated that Ad5wt had more capability in replicating its genome and producing virions than Ad5dlE1B 55kDa. This result was correlated to the high CPE that was observed in Ad5wt-infected cells compared to Ad5dlE1B 55kDa-infected cells as shown in Figure 1-b. One consideration could be the effect of E1B 55kDa deletion on the viral replication efficiency of Ad5dlE1B 55kDa, which is the more likely reason. Another possibility could be the presence of an inhibitory effect that can be overcome by wild-type adenovirus, but not mutant adenovirus. The last reason, should, if both viruses replicated at the same levels in HeLa cells, be considered. However, this was not the case. Interestingly, when viruses have higher replication efficiency in H1299 cells than HeLa cells, this suggested that different p53 status could affect the replication of oncolytic adenovirus differently.

Therefore, In addition to p53 backgrounds, we also evaluated the replication efficiency of all viruses specifically Ad5dlE1B 55kDa in both H1299 and HeLa cell lines in the absence and presence of various levels of wild-type p53. Both cell lines were transfected with plasmids that expressed ectopic p53 and then infected with Ad5wt, AdGFP, and Ad5dlE1B 55kDa at 24 hours post-transfection. Since each virus differs regarding the presence or absence of essential viral genes such as E1A and E1B, different replication pattern between viruses and different replication starting points were obtained in H1299 cells. These observed differences are mainly ascribed to the expression of wild-type p53 that can directly interact with viral genes affecting viral production rate and efficiency.

Ad5wt had the fastest replication and achieved the greatest rate among the three viruses. It started replicating earlier than the other two viruses and reached the highest viral DNA levels at 72 hours post-infection (Figures 2-4). This higher efficiency was mainly due to the presence of E1B 55kDa gene, whereas the delayed replication of the other two mutant viruses (Ad5dlE1B 55kDa and AdGFP) was largely contributed to the absence of this gene that can counter p53 repression activity toward viral replication and spread. This conclusion means that Ad5wt had the ability to inactivate quickly and block p53 activity in favour of virus replication. As a result, p53 cannot induce apoptosis in response to viral infection, thus facilitating the E1B transformation of the H1299 cell through the aid of the E1A protein for the favorite of virus production. It is consistent with the previously reported binding of E1B 55kDa gene to p53 to block its activity for efficient wild-type adenovirus replication (10, 49). Some of these studies have suggested that E1B 55kDa has a transcriptional repressor domain that affects p53 activation activity (10, 49). Other studies have elicited the binding of E1B 55kDa/E4-orf6 complex to p53, which antagonizes p53-mediated transactivation (50, 51).

On the other hand, in the mutant viruses that lack E1B 55kDa, p53 was not blocked. Therefore, both viruses started to replicate in the late stage compared to Ad5wt. However, the replication efficiency of both mutant viruses was different. This variability can be mainly ascribed to the presence of E1A region in Ad5dlE1B 55kDa, leading to overcoming the restriction and augment its replication to levels similar to Ad5wt but to a lesser extent. Such finding was shown in the case of the negative control plasmid, whereas it was more restricted in presence of p53 expression. The latter was not surprising since, in the absence of E1B 55kDa, p53 usually takes advantage by promoting cellular apoptosis to stop the viral spread. In the case of AdGFP, the deletion of the entire E1 region (ΔE1) caused a severe defect in virus replication efficiency, leaving DNA levels stagnant with no increase in the presence or absence of p53 (Figure 3). The same result was demonstrated in a study that showed the importance of E1 region in E1-deleted adenovirus's replication ability and suggested that cell cycling has a role in this manner (52). These results confirm that expression of wild-type p53 has a significant effect in hindering viral replication of both mutant adenoviruses at various extents, with no effect on wild-type adenovirus.

To confirm this conclusion, a similar experiment was conducted in HeLa cells, which contain functionally inhibited wild-type p53 (46, 47). The replication phenotypes of the three viruses in HeLa cells were different, with the lowest replication efficiency of Ad5dlE1B 55kDa. This observed difference is mainly related to the presence of active p53 in either presence or absence of replicative viral genes. Both Ad5wt and AdGFP replicated at high levels, which were not surprising for Ad5wt since it possesses a particular mechanism that blocks wild-type p53 function to promote cell cycle progression and facilitates its replication (Figure 5). The fact that E1B 55kDa functions as p53-inhibiting protein (9, 49) explain the variability of Ad5wt replication efficiency at the first measured time points. What supports this explanation is that p53 expression reached its peak at 2 and 24 hours post-infection and then started to decrease until it reached the cellular basal. These observations confirm that Ad5wt can inhibit p53 activity, and the existence of p53 in the cell has no effect on the virus's primary goal, which is to control the cell cycle mainly at the S phase towards viral DNA replication using E1A and E1B proteins (9, 18, 49).

The E1A gene was reported in some studies for its ability to overcome the degradation effect of HPV E6 on p53 in HeLa cells by stabilizing the p53 function through a phosphorylation process, as part of its function in the absence of E1B 55kDa (53-55). Also, it implements p53 dependent apoptosis in the absence of the entire E1B region (55). These facts explain why Ad5dlE1B 55kDa could not promote the replication under these conditions, and consequently, viral DNA levels started to decrease at two hours post-infection (Figure 7). The action of E1A in blocking the effect of HPV E6 and restoring or stabilizing endogenous p53 function has led to obtaining active p53, which therefore was able to hinder severely the replication of Ad5dlE1B 55kDa in conditions transfected with the test plasmids as well as the negative control plasmid. This assumption is supported by the higher replication of viruses that lack the entire E1 region as well as the accumulation of mutant p53 in the case of Ad5wt and AdGFP infections compared to the normal cellular wild-type p53 levels under Ad5dlE1B 55kDa infection. This finding reveals that the effect of HPV E6 was inhibited and p53 activity status returned to the normal levels otherwise p53 levels would have stayed the same across all the viruses. These results reconfirm that active p53 has a significant effect in suppressing Ad5dlE1B 55kDa replication but not the wild-type adenovirus.

If considering the hypothesis that different amounts of p53 can affect viral replication efficiency at various degrees (30), then different viral DNA levels should be obtained under variable levels of p53. In spite of this hypothesis, the different p53 levels that were expressed from the different expression plasmids in H1299 cells had no significant effect on the growth rate of all viruses (Figures 2-4). Both Ad5wt and AdGFP were either replicating or not replicating, respectively, in presence or absence of p53. The reason for these results in both cases was stated above, but the replication rate of each virus was the same under varying p53 levels that were expressed from the different p53-expressing plasmids (pA to pE). On the other hand, Ad5dlE1B 55kDa replicated more efficiently at 48 hours post-infection in p53 levels obtained from plasmid pB, than the other plasmids. However, this higher replication quickly disappeared at 72 hours post-infection, and the viral replication rate reached the same levels of the other plasmids. This outcome supports the results obtained from Ad5wt and AdGFP, and suggests that the effect of p53 is likely not to be dependent on its amount, but rather dependent on its activity which could be the critical key to the p53 effect.

Similar results were obtained in HeLa cells, as varying levels of p53 had no significant effect on viral replication rate. Such finding was observed with both Ad5wt and AdGFP regardless of the starting replication time. Additionally, Ad5dlE1B 55kDa replication reached zero levels in all conditions (Figures 5-7). This agreement in results on the p53 differing levels has led to the same conclusion in which the oncolytic Ad5dlE1B 55kDa replication efficiency may be based on the activity of p53 but not the quantity, which has not been assessed.

In conclusion, the replication efficiency of oncolytic Ad5dlE1B 55kDa mutant is more likely to be contributed to the status of cellular p53 rather than the cellular phenotype or the quantity of expressed p53. This result confirms the importance of functional p53 activity examination in different cancers to obtain a better understanding of the relationship between oncolytic Ad5dlE1B 55kDa and p53. Thus a clear vision would then exist for further improvement of the adenoviral virotherapy.

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Interest conflict
The authors have declared that no conflict of interest exists.

Author's contribution
Basma Abbas: study design, acquisition, analysis and interpretation of data and article writing.
Mohamed El-Mogy: participated in study design, analysis and interpretation of data and article writing.
Yousef Haj-Ahmad: participated in design and interpretation of data and final article revision.

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