Role of E4Orf3 Protein in adenovirus Life cycle in correlatin with E1B55k and Promyelocytic Leukemia protein Isoform II

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Abstract

Promyelocytic leukemia protein isoform II (PML-II) mRNA expression was interfered with anti-PML-II- specific mRNA to downregulate its expression to analyse the infection outcome in presence or absence of PML-II. PML-II transient knockdown gave an effective down regulation at the protein level then the transfected cells were challenged with adenovirus wild type (wt300) or Orf3- deleted mutant (InOrf3) to analyze the outcome of both viruses infection. DNA binding protein (DBP) expression increased in both types of infection with the removal of PML-II with more or less level of infection in both types of infection. Adenovirus yield was higher with the removal of PML-II in both types of wt or InOrf3 infection. Interferon treatment reduced the expression of early protein (E1b55k) in InOrf3 infection but not in wt infection when PML-II was absent. To conclude, E4Orf3 removal reduces the virus yield at later time points of infection through effect on E1b55k in an interferon induced environment.

Key words: E4Orf3, adenovirus, pml-II, E1b55k.

1. Introduction

Adenovirus infection is a worldwide infection and it is reemerging now in Asia and it represents about 69% of the hospitalized patients with few cases led to death. Adenovirus genome can persist in cells as an integrated genome or separate DNA material [1,2].Adenovirus early region encodes for many early proteins that are implicated in (DNA replication such as DBP [3].or antagonizing the innate immune response such as E4Orf3. Type I and type II interferon are triggered due to the Ad-IFN- gamma gene transfer [4]. Adenovirus E1A protein can block both type I and type II interferon, E1A stops the synthesis of IRF-1 mRNA due to the failure in phosphorylating the STAT1 alpha protein [5].In contrast to adenovirus wild type the mutant deficient for VAI RNA which cannot

produce RNA will not cope with the triggered interferon response as the VAI inhibits the latent P1eIF-2alpha kinase at late stage of infection [6].

Adenovirus is implicated in a specific interaction with anti-tumour and antiviral promyelocytic leukaemia proteins PML . Adenovirus type 5 protein IX disrupts the PML nuclear bodies in order to neutralize the antiviral effect of PML to ensure successful viral proliferation [7].An important role of PML and SP100 proteins was described for the correct assembly and crystallization of the late structural adenovirus proteins in fiber-deleted mutants as the non-used hexon and penton accumulated by PML and sp100 [8].DNA binding protein (DBP) protein directly targets the PML-NB but not the incoming viral genome which means that the viral proteins targets the PML-NB but not vice versa to support a conductive viral replication [9].At the transcription level, E1A-12S and E+1A13S which are responsible for the early viral transcription interact specifically with PML-II isoform through the conserved region 3 CR3 to enhance the transcriptional level of the viral protein [10].E4Orf3 is an essential to regulate the virus late gene expression as the latter diminished when the E4Orf3 region is mutated. In terms of plaques formation, InORF3 mutants produced plaques less than 10 of that of the wild types [11]. Adenovirus infection redistributes the PML into fiber-like structure due to the production of E4 Orf3 which concentrate with them into PML-NB [12]. During early adenovirus infection E1 and E4 region products associate with PML-NB and both sp100 and NDP55 leaves the adenovirus late transcription and early replication centers. Namely, such change is attributed to the effect of E4Orf3 as mutants lack to this protein fail in shutting off the cellular transcription machine and assembly of late viral mRNA . Both interferon treatment or PML overexpression can completely block the PML-NB reorganization which are the target of early viral gene products [13].

Orf3 protein interacts with PML-II through 40 aa segment within C- terminus specific region of PML-II [14].Recently, it was found that E4orf3 works as a viral ubiquitin- like modifier (SUMO) to achieve substrate sumoylation of substrate for proteasomal degradation [15]. So, through such activity of E4Orf3, TFIII protein level is decreased due to the sumoylation of the latter by E4Orf3 blocking by that function of an important cellular transcription factor to ensure successful life cycle [16]. In addition to that, E4Orf3 modifies about 51 proteins to modulate the cellular immune response and sub-nuclear re-localization [17].It targets the PIAS3 which a cellular E3 SUMO ligase and such action is conserved over different adenovirus types to ease the virus replication [18].

E1b55k is a transcription repressor of p53 related genes through interacting with RNA polymerase [19]. E1b55k blocks the interferon response to enable the virus to form the replication centers [20]. So the idea is to investigate whether removing InOrf3 can compromise the expression of E1b55k in an IFN- upregulated environment and would that affect the virus yield of both wild type and InOrf3 mutant.

2. Materials and Methods

Stable transduced HeLa cells

Permanent PML-II depleted cells and control GFP cells were produced by genetic modification using the lentiviral system to deliver the anti-PML and GFP constructs into HeLa cells genome to investigate the function of PML-II gene in adenovirus life cycle [21].

Cells extraction and Western-blotting

Cells were lysed by (1x SB of Laemmli sample buffer2x (4 ml of 10% SDS, 2 ml of 20% glycerol, 1.2 ml of 1 M Tris-HCl pH 6.8 2.8 ml of H2O and 0.02% w/v of bromophenol blue). Samples were loaded onto 8% polyacrylamide gels after 10 minutes boiling and electrophoresed at 70 V for 4 hours. Proteins were then transferred to nitrocellulose membrane (10 cim ×7 cim) at 0.35 A for 1.5 h as a quick transfer in a cold room. Membranes were blocked for 1h at room temperature with 2% blocking reagent in PBS/0.05% Tween 20 (PBS-T), 62. Fllowed by 1 hr incubation with anti- PML- antibodies or anti E1b55k at dilutions of 1:50k or 1:250 respectively.

Adenovirus infection

Confocal Immunofluorescence

Cells were plated at r 1×10^5/ well culture in 12- well plates contain sterile coverslips to analyse the outcome of infection after transient depletion of PML-II or PML-V as a control 48 hours later, cells were fixed with 10% formalin and permeabilised with 0.5% NP40 in PBS with washing after each step. Blocking was achieved by 1% bovine serum albumin (BSA) in PBS for 45 minutes. After 3 washes with PBS, 1:250 dilution of DBP primary antibodies were added and left for 45 minutes. the primary antibodies were replaced with diluted 250 µl of Alexafluor 488 secondary antibodies after 3 washes with PBS for another 45 minutes. Coverslips were washed twice with PBS. Nuclei were stained with 1 µg/ml DAPI for 5 minutes. Coverslips were mounted with Vectasheld mounting medium. The

images were taken as single optical section by a Leica Leica SP5 system and Leica software.

Table 1. Types of adenoviruses were used in this study

Virus	Description	References
Wt300	Wild type Ad5	[22]
InOrf3	Implanting of E4 frame-shift (300 backbone)	[23]

Table 2. Primary and secondary antibodies were used in this study

Abs	Description	Western blotting	Immunofluorescence	FACS	References
Primary antibodies					
Mouse anti-E1B55	Detects 1 E1B55K expression	1/250	-		[24]
Mouse anti DBP	Detects Ad5 DNA binding protein	1/10000	-	1/5	[25]
Secondary antibodies					
Goat anti-Mouse IgG	Horse Radish peroxidase conjugate	1/10000			sigma
Goat anti-Mouse IgG (H+L)	AlexaFluor 488(conjugate		1/500	1/50	Technology

membranes were incubated with the appropriate antibodies (Table 2.10) for 1 h. Membranes were washed 7 times for 13 minutes with PBS-T, then incubated with the appropriate secondary antibodies (Table 2.11). Finally, the reaction was detected by ECLTM Advance reagent according to the manufacturer's instructions by exposure to Fuji Super RX X-Ray film. Different loading controls were used in this study. Depending on the molecular mass of the protein (GAPDH, β-actin or α- tubulin) the loading control was either acquired by cutting the lower part of the same membrane or loading the same samples on a different gel

virus titration

Ad5 wt300 was or InOrf3 were tittered by counting DBP a fluorescence-positive cells as Flourescent Focus Assay (FFA). HeLa cells were plated at 1×10^5/ well in 24- well plates for 24 hours. Serial ten-fold dilution (10-1 to 10-10) of each virus was prepared in serum-free DMEM free serum. Grown cells were challenged with 50µl of each dilution as duplicate after removing the DMEM. Cells were incubated with the virus for 1 hour, then the virus was discarded and the cells were covered again with a fresh DMEM 10% FBS and incubated for 16 hours. Cells were then fixed and stained with anti-DBP antibodies and AlexaFluor 4888 secondary antibody as for confocal microscopy. The DBP foci were counted using UV- microscop

3. Results and discussion

With the aim of evaluating adenovirus early gene expression (both wild type and InOrf3 mutant) in presence or absence of PML-II, PML-II mRNA was interfered with PML-II siRNA by transient knockdown with parallel samples that were transfected with Ctrl siRNA only. It is obvious that the increased concentration of PML-II siRNA showed better inhibitory effect to the expression level of PML-II compared to the lowest concentration which showed the same expression intensity compared to Ctrl siRNA (fig.1). In fact, knockdown the PML mRNA is to some extent easy to be achieved and PML-II siRNA interferes with the expression of all PML isoforms and as t is shown in (Fig.1) and this comes consistently with what was published by [26,27]

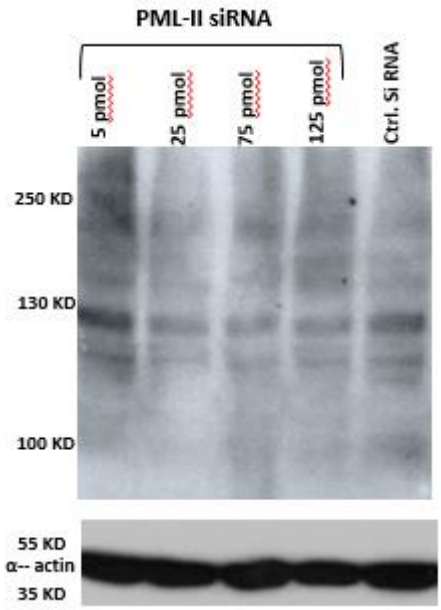


Figure.1. Transient knockdown of PML-II protein in HeLa cells. Hela cells were plated out at 12 well plate as at a density of 2.5×10^5 . 24 hours later, the cells were transfected with 5, 25, 75, and 125 pmol of PML-II siRNA and 125 pmol of ctrl siRNA for 48 hours . The transfected cells were lysed with SDS-lysis buffer to load them on polyacrylamide gel. The membranes were stained with anti-PML-II primary antibodies and then with anti- rabbit poly clonal antibodies.

The transfected cells were challenged with wt or inorf3 mutant at moi of 1 for 20 hours and the early stage of infection was examined by confocal imaging. Clearly the DBP expression was higher with the removal of PML-II compared to its level in cells were transfected with PML-V siRNA and then challenged with wt300 or InOrf3 infection (fig.2 & fig.3). PML is an interferon upregulate gene and its expression is induced with type I and type II interferon [28]

Many studies pointed to the imperative role of PML as DNA and RNA as antiviral such as Herpes simplex virus (HSV) type1, Epstein-Barr virus , Human cytomegalovirus (HCMV), adenovirus, human immune deficiency virus (HIV), Influenza virus [13, 28, 29, 30, 31, 32, 33]

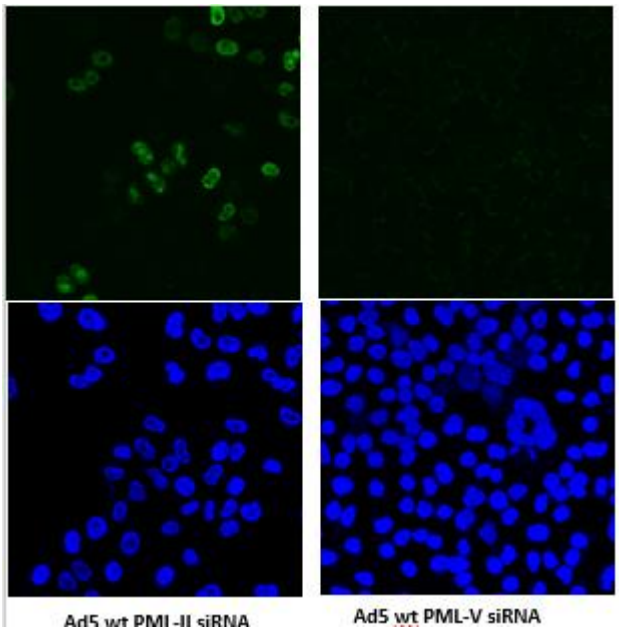


Figure2. DBP expression of adenovirus wt infection in HeLa cells. Hela cells were plated out on coverslips 12 well plate as at a density of 1×10^5 . 24 hours later, the cells were transfected with PML-II or PML-V or Ctrl. siRNA for 48 hours then all were infected with ad5wt for 8 hours. The transfected cells were fixed, permeablized and stained with ani-DBP monoclonal primary antibodies and then stained with Alexafluor-488 secondary antibodies, then images were taken by confocal microscopy.

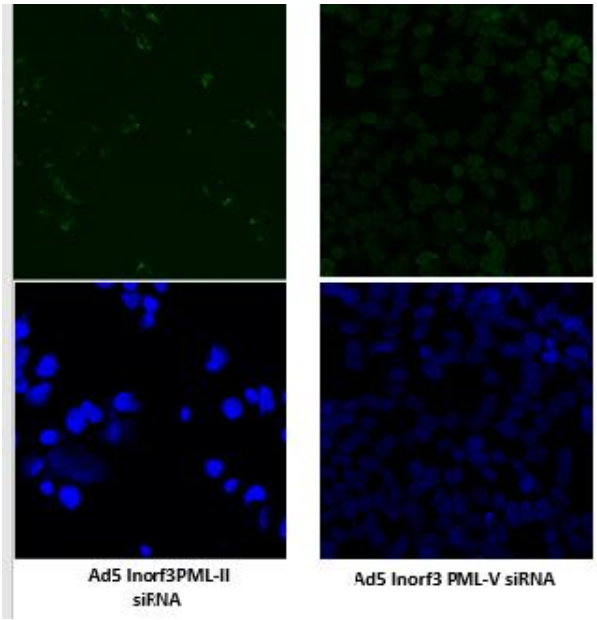
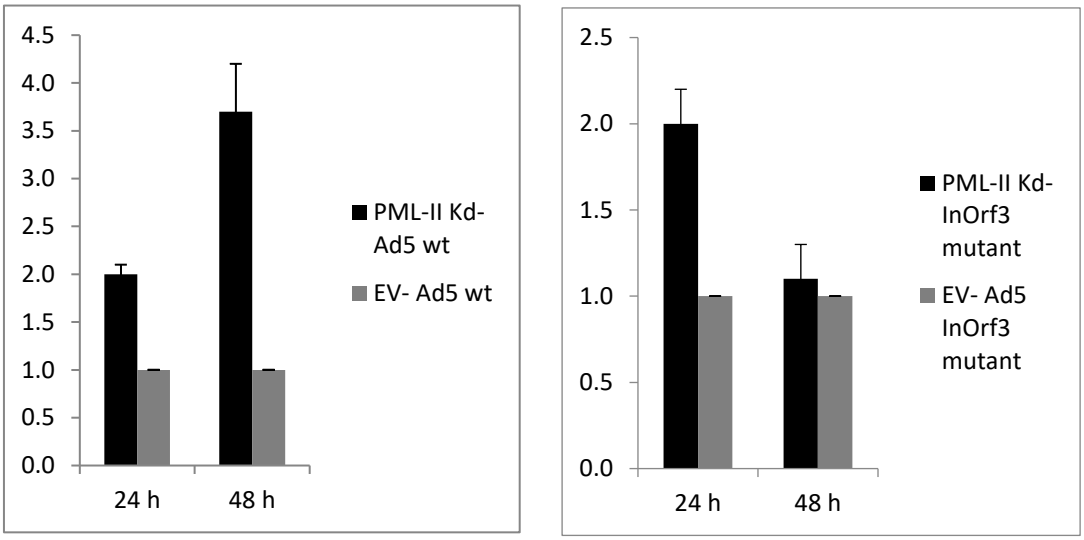


Figure3. DBP expression of Inorf3 infection in HeLa cells. Hela cells were plated out on coverslips 12 well plate as at a density of 1×10^5 . 24 hours later, the cells were transfected with PML-II or PML-V or Ctrl. siRNA for 48 hours then all were infected with Inorf3 for 8hours. The transfected cells were fixed, permeablized and stained with ani-DBP monoclonal primary antibodies and then stained with Alexafluor-488 secondary antibodies, then images were taken by confocal microscopy.

More specific effect was attributed to PML-II isoform which inhibits adenovirus infection partially due to IFN response and mainly due to the elevation in hsp70 expression [21]. More or less, the same result was obtained when cells were infected with Inorf3 mutant as the DBP fluorescence was expressively high with the reduced expression of PML-II (fig.2). Phenotypically InOrf3 mutant replicates as normal as the adenovirus wild type in vitro cell culture. Deleting E4 coding region including the Orf3 produces virus progeny as similar as the wild type with comparable level of cellular and humoral immune response in vaccinated mice.

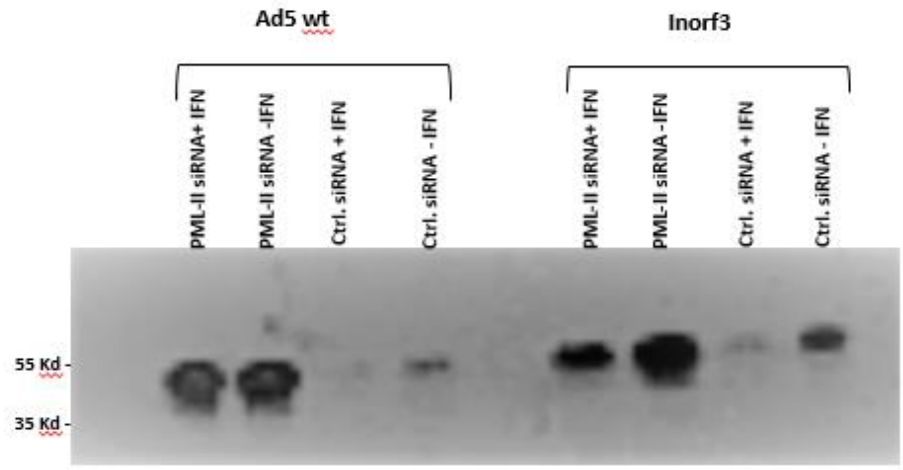
Virus yield

In order to evaluate the wt and InOrf3 virus yield, HeLa cells were infected with wt or InOrf3 and stained for DBP fluorescence counting. Clearly wt infection progressed better in PML-II Kd cells since the wt virs yield was two fold more in PML-II deprived cells compared to EV. The difference became sharper after 48 hours. PML-II kd cells showed an increase in virus yield reached more than 3 fold than in control samples (EV). Consistently, in the case of InOrf3, at 24 hours post infection the infection roughly showed 2 fold increase compared to the control. While the infection almost showed no difference between PML-II kd cells and EV cells when it was left to progress for additional 24 hours (fig.4). As previously stated the PML-II regulates the type I interferon response and adenovirus wild type infection progresses better when PML-II expression is transiently or permanently reduced. The wt300 adenovirus infection progressed better with the in PML-II knockdown cells partially due to the reduced type I interferon response or mainly due to the increased hsp70 in PML-II knockdown cells [21, 34]. Due to the fact that wild type adenovirus antagonizes the interferon response by several gene products such as E1A, E1B55k,VA RNA **I** [**6,20, 35**] in addition to E4Orf3 protein, InOrf3 mutant gave comparable virus yield as it counteracted the interferon response by those other means at 24 hour post infection in PML-II depleted cells.



Figuer.4. Ad5 and Inorf3 yield in PML-II Kd and EV cells. PML-II Kd and **EV cells were plated at a density of 1×10 /ml for 24 hours, infected at moi of 1 with Ad5wt300 or Inor3 for 24 and 48 hours. Samples were harvested and subjected to freeze-thaw cycles to release virus and then serial dilutions were prepared from each sample and used to infect standard HeLa cells for 16 hours. Cells were fixed and stained using anti-DBP monoclonal antibodies as primary Abs and 488 Alexa fluor goat anti mouse Abs as secondary antibodies. Samples were examined using UV-microscopy and DBP foci were counted by eye. Numbers from duplicate cultures were averaged and divided by cell numbers in the producer culture, then normalized to the yield in EV cells. A. Wt Virus yield at 24 hours. B. InOrf3 Virus yield at 48** hours.

InOrf3 mutant infection decreased at 48 hours post infection and that might belong to lack of Orf3 protein which affected the virus yield at later time points since the Orf3 associates with E1B55k in nuclear matrix and the former promoters. In fact, E4Orf3promots the replication of the virus in a background lacks the E1b55k [36, 37]Furthermore, the accumulation of adenovirus mRNA in nucleus and cytoplasm is significantly reduced with a concomitant mutation of E4Orf3 and E1b55k [38]. Prior treatment with IFN reduced the expression of E1B55 k in InOrf3 mutant infected cells but not in wt infected samples (Figure 5).



Figuer.5. E1b55K expression in Ad5wt or Inorf3 infected HeLa cells. HeLa cells were plated at a density of 1×10 /ml for 24 hours, then transfected with PML-II siRNA or Ctrl. siRNA at a concentration of 125 pmol for 48 hours. Cells were treated with 1000 /iU IFN later for 6 hours, then they were infected with Ad5wt or Inorf3 at moi of 1 for 20 hours. Samples were harvested and lysed with SDS-lysis buffer to load them on polyacrylamide gel. The membranes were probed with anti-E1b55K primary antibodies (1:100 dilution factor) and then stained with anti- mouse secondary antibodies (1:10000 dilution factor.

Such data link the both gene products in the way that they interact with the cellular immune response and their roles in adenovirus life cycle in terms of localization and function. E4Orf3 together with E1B55k transform baby rat kidney cells and E4Orf3 colocalizes to PML-NBs in transformed cells so its oncologic activities is linked to E1b55k and PML-NBs independent from p53. Triggering the PML-NBs redistribution promotes the oncolytic processes in cooperation with viral proteins such as E4Orf3 [34]. Recently, a new role of E4Orf3 in regulating the cellular transcription machinery is revealed including the signal transduction and cellular defense and PML track formation is necessary for that function [39]. E4Orf3 expression reduces the increased IFN-ß due to the stimulation with truncated form of PML-II [34]. In conclusion, in an interferon stimulated environment virus yield is diminished with removing the E4Orf3 through an effect on E1b55k expressin.

Aknowledgment

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دور بروتين اطار الشفرة المفتوحة المبكر في دورة حياة فيروس الحليمات الغدية خلال التاثير على البروتين الفيروسي المولد للاورام و على بروتينات ما قبل النسيلة الصيغة الثانية الخلوية

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المستخلص

تم التعارض مع الحامض النووي المراسل لبروتين النسيلة الطراز الثانيـ PML-II بشكل موقت او دائمي لغرض دراسة تاثيره على دورة حياة الفيوس ذو الطراز البري والفاقد للـ E4Orf3 . لوحظ ان لاالتعارض مع التعبير الجيني للـ PML-II كان فعالا اذ تم تخفيض انتاج البروتين. اما فيما يخص الجانب الفيروسي لوحظ ارتفاع التعبير الجيني للبوتين المرتبط بالدنDNA binding protein DBP في حالة الاصابة بالنوعين من الفيروسات عند تخفيض انتاج الـ PML-II اختلف انتاج الفيروس Virus yield اختلف في العينات المصابة بالفيروس ذو الطراز البري عنه في الطراز المطفر اذا لوحظ ارتفاع مستوى انتاج الفيروس في الحالة الاولى عند خفيض انتاج ال PML-II بعد 24 ساعة والذي تعزز بعد 48 ساعة من الاصابة . اما في الحالة الثانية فقد لوحظ ارتفاع انتاج الفيروس المطفر بعد 24 ساعة عند خفيض انتاج ال PML-II الا انه انخفض مستوى الانتاج بعد 48 ساعة وقد يعود ذلك ايضا الى تاثر البروتين المبكر E1b55k بالمعاملة بالانترفيرون في حالة الفيروس المطفر. ويمكن الاستنتاج بان ابعاد الـ E4Orf3 يخفض انتاج الفيروس في مراحل متاخرة من الاصابة من خلال تاثير الانترفيرون المحفز على التعبير الجيني لبروتين الـ E1b55k .