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The activation of p53 mediated by Epstein-Barr virus latent membrane protein 1 in SV40 large T-antigen transformed cells

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Abstract The Epstein-Barr virus (EBV) encoded latent membrane protein 1 (LMP1) plays an important role in the carcinogenesis of nasopharyngeal carcinoma (NPC). The tumor suppressor p53 is an important transcription factor. The mutation of the p53 gene is the frequent alteration in most of tumors, but nearly 100% wild-type p53 gene is found in NPC biopsy. Here, our study testified that SV40 T-antigen transformed nasopharyngeal epithelial cells contained free, wild-type p53. Moreover, LMP1 regulated p53 both at transcriptional and translational level. Furthermore, the mechanism of p53 accumulation mediated by LMP1 from post-translational level-phosphorylation and ubiquitination were determined. Therefore, the effects of EBV LMP1 on p53 may potentially contribute to EBV-associated pathogenesis.

Keywords: Epstein-Barr virus; Latent membrane protein 1; p53; SV40 T-antigen

1. Introduction

Nasopharyngeal carcinoma (NPC) is the high-frequency cancer in China, Southeast Asia, some western countries [1]. In contrast to other head and neck cancers, a unique feature of NPC is its strong association with Epstein-Barr Virus (EBV). Among the EBV encoded genes, latent membrane protein 1 (LMP1) is the only one with oncogenic properties [2]. LMP1 can transform established rodent cell lines and alter the phenotype of both lymphoid and epithelial cells, also it causes epidermal hyperplasia, lymphomas and papillomatosis in transgenic mice [3,4]. The tumor suppressor p53 is an important transcription factor that evolved in cell cycle arrest, apoptosis, DNA repair, or senesce via inducing the different subsets of genes. Meanwhile, the activation of p53 is need to be modified at post-translational level, including phosphorylation, ubiquitination and acetylation events, and leads to temporary growth arrest or apoptosis [5].

Alteration of the p53 gene, such as point mutation, deletion, or rearrangement, are found in most of the human tumors, which contribute to the complex network of molecular events leading to tumor formation. Among them, the mutation of the p53 gene is the frequent alteration in the carcinogenesis of cancers [6]. It is reported that the mutations of p53 are found clustered in four "hot-spot" located in exons 5, 7, and 8 within the DNA-binding domain, which coincide with the four most highly conserved regions of the gene [7]. Another important mechanism of p53 inactivation in the tumors is that p53 protein can form a complex with several different DNA tumor virus genes and produce cell transformation, including the simian virus 40 large tumor antigen [8], the E1B 55 kDa protein form adenovirus type 5 [9], and human papilloma virus types 16 and 18 E6 protein [10].

Unlike other human tumors and Burkitt lymphoma, another EBV-associated malignancy, nearly 100% wild-type p53 gene is found in NPC [11–13]. Therefore, p53 mutation is an infrequent event and maybe has some significant role in the pathogenesis of NPC. EBV infection and the over-expression of p53 are involved in the multi-step process of human nasopharyngeal epithelial carcinogenesis [14,15]. p53 over-expression seemed to occur at an early stage in the development of NPC and associated with advanced disease stage, poor response to therapy [16,17]. But whether p53 has its biological role in the carcinogenesis of NPC is still disputed.

Using immortalized normal nasopharyngeal epithelial cell lines will allow us to characterize the functions of EBV gene in this specific host environment [18]. Despite that wild-type p53 existed in most of NPC biopsy were confirmation, however, p53 mutation in "hot-spot" is frequently observed in cell lines derived from the primary NPC tumors [19]. So the immortalized normal nasopharyngeal cell line (SV40 T-antigen transformed) with no p53 mutation will provide a good model to study the regulation of EBV to p53 in the carcinogenesis of NPC. Since the stability of p53 is increased in SV40-transformed cells, we hypothesized that in SV40-transformed nasopharyngeal epithelial cell, there existed lot of active, wild-type p53, not complexed to larger T. Furthermore, the activation of p53 by LMP1 in this model will be confirmation.

2. Methods and materials

2.1. Cell culture, plasmids and reagent

NP69, NP69-pLNSX, NP69-LMP1 cells (NP cells) were propagated in the defined Kertinocyte-SFM (KSFM, GIBCO, Life Technologies, Basel, Switzerland) supplemented with growth factors, and maintained

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at 37 °C with 5% CO_2. The parent cell line NP69 is a SV40 T-antigen transformed line, the characters were described as previous [18]. The B95.8 cell line (ATCC CRL-1612) was grown in RPMI 1640 culture medium (GIBCO-BRL) supplemented with 10% feral bovine serum. The Medium was supplemented with 10 mg/ml streptomycin and 10 IU/ml penicillin. pSG5 and pSG5 B95.8-LMP1 had been previously described. Effectene Transfection Reagent (QIAGEN Company, Hilden, Germany) and Luciferase Assay System were used (Promega corporation, Madison, WI, USA).

2.2. RT-PCR

Total RNA was extracted from NP69, NP69-pLNSX and NP69-LMP1 cells using Trizol solution according to the manufacturer's recommendation (invitrogen). Single-stranded cDNAs were synthesized by M-MLV reverse transcriptase (invitrogen) and amplified by PCR. The primer sequences for the p53 entire coding region (P1/P2) and four mutant hot-spot regions (SHS01/SHS02, SHS03/SHS04) were described previously [13]. For internal control, the *actin* primer sequences were (Sence) 5′-TTCCAGCCTTCCTTCCTGGGG-3′ and (antisence) GCTCAGGAGGAGCAAT containing 200 bp in between two primers with annealing temperature 55 °C and 35 PCR cycles.

2.3. Immunoblotting analysis

Cells were harvested by centrifugation and resuspended in lysis buffer containing 20 mM HEPES, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 1% Nonidet P-40, phosphatase inhibitor mixture, and protease inhibitor mixture. Cell pellets were sonicated briefly and cell debris sedimented by brief centrifugation at 13000 rpm for 5 min at 4 °C. Supernatants were transferred to fresh tubes, and protein content was determined by the Bradford assay (Bio-Rad, USA). For Western analysis, 50 μg of total protein was loaded onto 8–12% Tris–glycine polyacrylamide gels and subjected to electrophoresis. Proteins were visualized by ECL chemiluminescence reagents (Pierce Chemical Co., Rockford, IL, USA) using primary antibodies specific for human p53 (SC-126; Santa Cruz, CA, USA), phospho-p53 Ser20 (SC-18079; Santa Cruz, CA,USA), Ser392 (SC-7997; Santa Cruz, CA, USA), p21 (SC-756; Santa Cruz, CA, USA), MDM2 (SC-965; Santa Cruz, CA, USA), phospho-p53 Ser15 (9284), Thr81 (2676) were from Cellular Signaling Technology (Beverly, MA), LMP1 (CS 1-4, DAKO) and α-tubulin (Sc-5286), goat anti-rabbit IgG-HRP (SC-2004), goat anti-mouse IgG-HRP (SC-2005) and donkey anti-goat IgG-HRP (SC-2020) were from Santa Cruz (CA, USA).

2.4. Immunofluorescence staining and confocal microscopy

Cells were cultured on glass coverslips. After they were washed with PBS, cells were fixed with ice methanol for 20 min. To display endogenous p53 protein, cells were first incubated with p53, and then reacted with corresponding FITC-conjugated IgG as secondary antibody. Cellular nuclear were stained with propidium iodide (PI). p53 and PI were visualized under a confocal microscope (Bio-Rad).

2.5. Assay for p53-dependent transcription activity

p53-dependent transcription activity was assayed by using NP cells line transiently transfect a luciferase reporter gene controlled by p53 DNA binding sequences. Confluent monolayers of the NP cells in 24-well plate were trypsinized. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 until the cells were 80–90% confluent. The cells were extracted with lysis buffer RLB (Promega), and luciferase activity was measured using a luminometer (Promega). The results were expressed as relative p53 activity.

2.6. Sequential immunoprecipitation

Sequential immunoprecipitation of immunodepleted extracts was performed in the previous procedure. Aliquotes of the clarified lysate were reacted for overnight at 4 °C with the first antibody, T-Ag or p53 antibody, before addition of protein A-Sepharose beads for an additional 2 h. Following brief low-speed centrifugation to pellet the beads, the supernate was reimmunoprecipitated with the T-Ag or p53 antibody in an identical manner. This precleared extract was then aliquoted for immunoprecipitation with one of the two remaining antibodies. The immunoprecipitated protein were then separated in 8% SDS–polyacrylamide gels and subjected to electrophoresis. Proteins were visualized by ECL chemiluminescence reagents.

3. Results and discussion

3.1. Free, wild-type p53 in SV40 T-antigen transformed nasopharyngeal epithelial cells

Firstly, immunoblotting analysis was used to show the expression of SV40 T-antigen in LMP1 negative and positive cells. As shown in Fig. 1, LMP1 failed to alter the expression level of SV40 T-antigen in these cells. Next, p53 that unbound to T-antigen would be detected by Western analysis of sequential immunoprecipitation (Fig. 2). After determined that lots of free p53 existed in NP cell, wild-type or mutant-type p53 were detected in these cells. As shown in Fig. 3, these cell lines generated fragments of 1.3 kilobases (kb), the size expected for the wild-type open reading frame with reverse transcriptase-PCR (Fig. 3A). Next, fragments of mutation hot-spot regions, showing the expected sizes of 250 and 272 base pairs (bp) according to wild-type sequence, were determined (Fig. 3B). Sequencing data showed that there was at least no point mutation in four "hot-spot" (data not show). Taken together, these results for the first time suggest that there are large amounts of free, wild-type p53 in SV40 T-antigen transformed nasopharyngeal epithelial cells.

The previous concept consistently considered that SV40 T-antigen is contributed to tumourigenesis due to continuous inactivation of p53 [20]. But, the recent report argued the above studies that p53 is constitutively active in cells transformed with the SV40 T-antigen. These findings indicated that activated p53 is unbound to T-antigen in SV40 T-antigen transformed cells, but are metabolically stable, like p53 in non-SV40 T-antigen transformed cell [21]. Moreover, DNA damage could induce p53's activity in SV40 T-antigen transformed cells [22]. Beside with SV 40 T-antigen, in human papillomavirus- transformed cells and adenovirus E1-transformed cells, basal DNA-damage-inducible p53 functions are also intact [23]. Here, we provide the similar evidences that there are lots of free p53, unbound to SV40 T-antigen, in SV40 T-antigen transformed NP cells. This raises the possibility that transient inactivation of p53 via SV40 T-antigen is responsible for malignant transformation in some stage of tumorigenesis, but not permanent inactivation of p53.

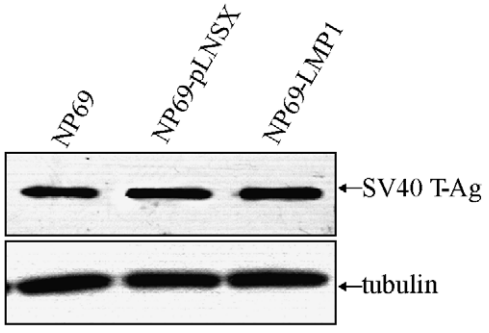

Fig. 1. Immunoblotting analysis of the expression of SV40 T-Ag in NP69, NP69-pLNSX and NP69-LMP1 cells. α-Tubulin as an internal control.

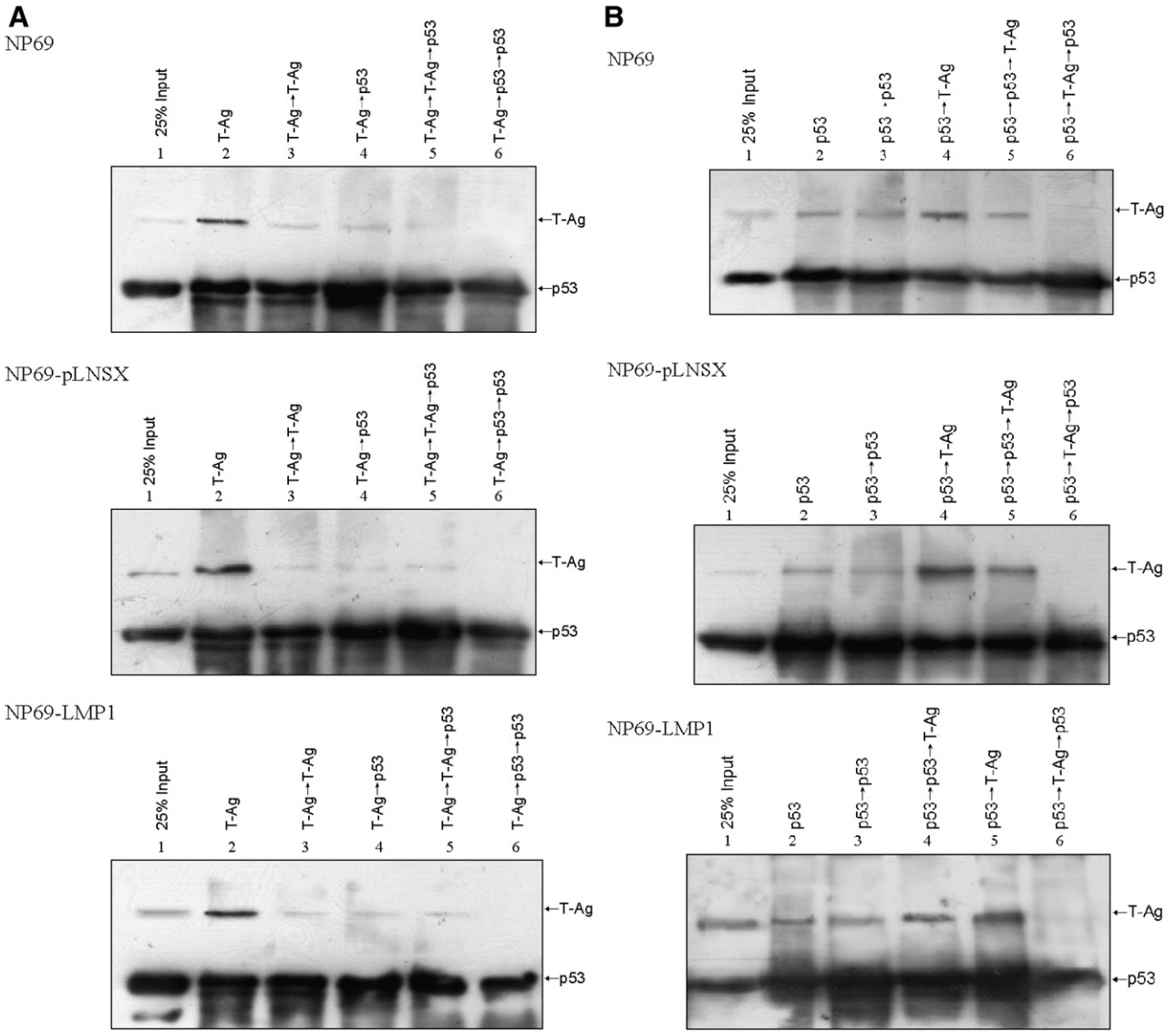

Fig. 2. Sequential immunoprecipitation of T-Ag and p53. (A) Extracts from wt T-Ag transformed nasopharyngeal cells propagated at 37 °C were sequential immunoprecipitation. (B) Extracts from wt p53 transformed nasopharyngeal cells propagated at 37 °C were sequential immunoprecipitation. The immunoprecipitates were then subjected to immunoblotting analysis and probed with T-Ag and p53 monoclonal antibodies.

3.2. Ionizing radiation-induced p53 activation in NP cells

Free, wild-type p53 is very sensitive to IR or UV-induced DNA damages, followed by initiating the expression of down-stream genes. To confirm intact p53 being in NP cells, cells were treated with 5 Gy of IR. We collected the cells irradiated 3 or 6 h by 5 Gy and analyzed the expression of p53, MDM2 and p21. As expected, IR induced the accumulation of p53, MDM2 and p21 at both 3 h and 6 h, which indicated the activation of wild-type p53 in NP cells. Interestingly, the levels of p53, MDM2 and p21 induced by LMP1 were less than the levels initiated by irradiation (Fig. 4A). It is reported that IR increased the p53 expression levels in wild-type p53 cells, however, the p53 level remains unchanged in cells with mutant p53 during the same post-irradiation period [24]. Maybe T-antigen is a highly multifunctional protein that acts on tumorigenesis regulation by various know and unknown pathway, at least inactivation of p53 is only the transient events in carcinogenesis.

Among the post-translational modification of p53, phosphorylation has been studied most intensively and has been proposed to play a critical role in the stabilization and activation of p53. Next, the phosphorylation of p53 in IR treated NP cell were measured by p53 specific-phosphorylated antibody. Our data showed that IR induced the phosphorylation of p53 at Ser15, Ser20, Ser392 and Thr81, less than that of LMP1 induced phosphorylation of p53 (Fig. 4B). Besides with LMP1, Epstein-Barr virus nuclear antigen 1 (EBNA1) [25], hepatitis C virus core protein [26] and HIV-1 envelop [27] also can modulate p53 in post-translational level. Furthermore, in the following study we confirmed that LMP1-induced phosphorylation of p53 at Ser15 was directly by ERKs; at Ser20 and Thr81 by JNK, at Ser15 and Ser392 by p38 kinase. The phosphorylation of p53 was associated with its transcriptional activity and stability modulated by LMP1 [28]. These data emphasized that the free, wild-type p53 existed in NP cells again from IR treatment and post-translational level. SV40

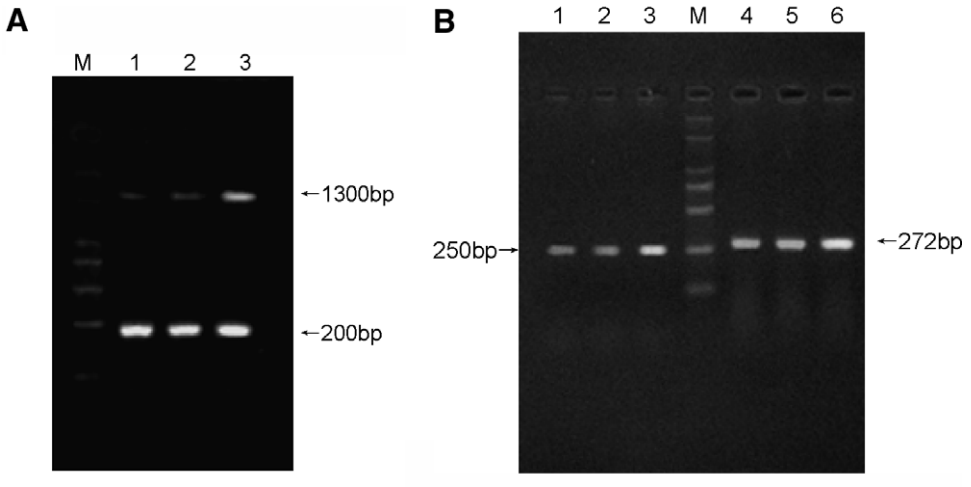

Fig. 3. Expression of p53 mRNA in NP cells. Total RNAs were extracted from NP69, NP69-pLNSX and NP69-LMP1 cells, reversed-transcribed into cDNAs, and amplified by PCR using two pairs of primers. DL2000 DNA marker was lane M. Samples of the PCR products were analyzed on 1% agarose gels by using the associated pairs of PCR primers. (A) Lanes 2–4 was from RNA of NP69, NP69-pLNSX and NP69-LMP1, respectively. P1 and P2 generate 1300 bp fragment, actin primer generate 200 bp fragment. (B) Lanes 1 and 4 were from RNA of NP69, lanes 2 and 5 were from RNA of NP69-pLNSX, lanes 3 and 6 were from RNA of NP69-LMP1. SH01/SH02 generate the 250 bp fragment (lanes 1, 2 and 3); and SH03/SH04 generate 272 bp fragment (lanes 4, 5 and 6).

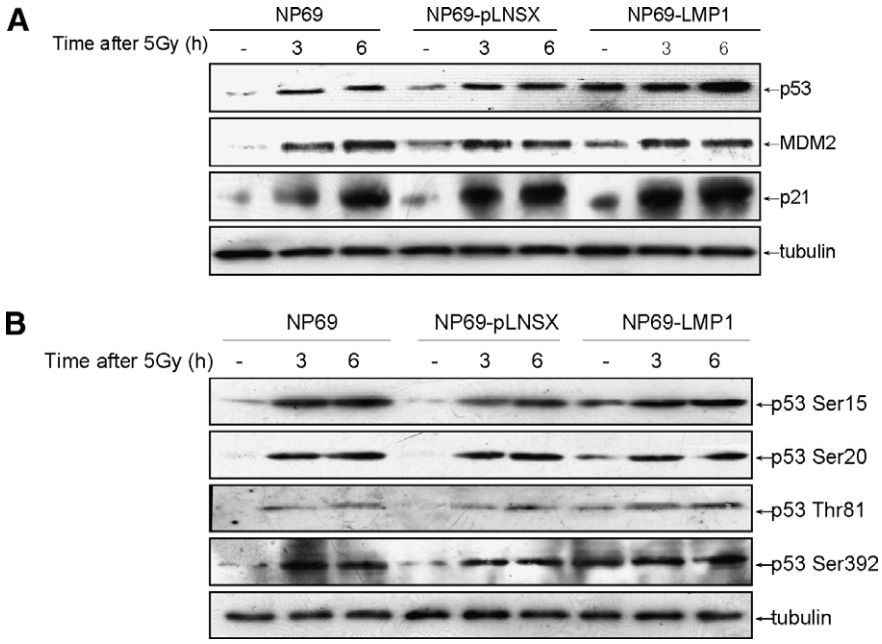

Fig. 4. Ionizing radiation (IR)-induced the activation of p53. (A) p53, MDM2 and p21 protein expression in NP69, NP69-pLNSX and NP69-LMP1 cells. (B) The phosphorylation of p53 Ser15, Ser20, Ser392 and Thr81 in NP69, NP69-pLNSX and NP69-LMP1 cells. α-Tubulin protein level was detected as a loading control.

T-antigen has no influence in function of p53 in the carcinogenesis of EBV LMP1.

3.3. Activation of p53 regulated by EBV LMP1 in NP cells

As an important transcription factor, p53 executes its function of transcription factor in the nucleus. To test whether p53 was retained by LMP1 in the nucleus, p53 was immunostained in SV40 T-antigen transformed cells. As shown in Fig. 5A, in LMP1 negative cells, p53 mainly located in the nucleus, cytoplasmic part was also showed the expression of p53. However, in NP69-LMP1 cells, we observed that cytoplasmic staining of endogenous wild-type p53 was little. Next, NP cells were subjected to subcellular fractionation in order to confirm the nuclear accumulation of p53 induced by LMP1. These data showed that the cytoplasmic and nuclear levels of p53 were increased mediated by LMP1, and the nuclear p53 level of NP69-LMP1 cells was more obvious. The purity of the nuclear and cytoplasmic fractions was verified by immunoblotting with

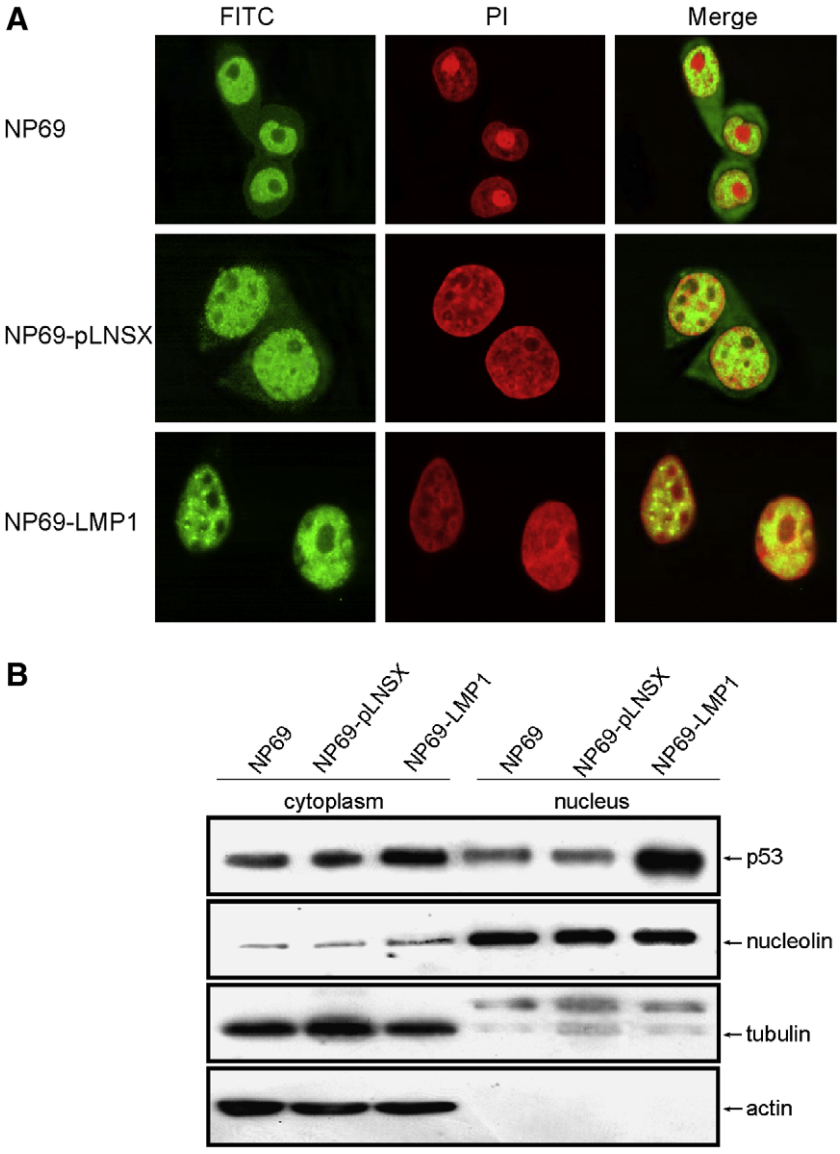

Fig. 5. Localization of p53 in NP cells. (A) Cells immunostained with anti-p53 antibody followed by the corresponding FITC-conjugated anti-IgG secondary antibody to show p53 protein. Simultaneously, the cells were stained with propidium iodide to display the nuclei. (B) The expression of p53 in cytoplasmic and nuclear parts among these cells. Cytoplasmic and nuclear extracts were prepared and immunoblotting analysis of the expression level of p53. α-Tubulin, nucleolin and actin were used as markers to check the extent of nuclear and cytoplasmic separation.

antibodies against nucleolin, α-tubulin and actin, respectively (Fig. 5B). These findings suggest that LMP1 could induce the nuclear accumulation of p53.

Since the transcriptional activity of p53 is important for p53-mediated nucleotide excision repair (NER), we investigated whether EBV LMP1 promote the transcriptional activity of p53. Reporter containing three consensus p53-binding sites fused to luciferase gene was transfected into NP cells. In the presence of LMP1, p53 transactivation activity was increased, compared to the NP69 and NP69-pLNSX cells (Fig. 6A). Furthermore, the protein expression level of LMP1, p53 and downstream genes in NP cells were analyzed. The expression of LMP1 in B95.8 cells, an EBV positive cell line, was as positive control. Our data showed that LMP1 could increase the amounts of p53, MDM2 and p21 (Fig. 6B). To better understand how p53 is regulated in LMP1-stressed cells, we measured the expression of these proteins through transiently transfected LMP1 to NP69 cells. Immunoblotting revealed that LMP1 could obviously up-regulate the protein level of p53, MDM2 and p21, moreover, LMP1 was only expressed in NP69-pSG5LMP1 cells, which assured the efficiency of transfection (Fig. 6C).

p53 increases MDM2 at the transcriptional level, but MDM2 suppresses p53 activity at the post-translational level. MDM2 is an E3 ubiquitin ligase that directs the ubiquitination of p53 via 26 S proteasome, resulting in the degradation of p53 [29]. In the previous study, the phosphorylation of p53 mediated by LMP1 prevented the interaction of MDM2 and p53 through MAP kinases was confirmed [28]. To evaluate these observations further, we treated these cells in the presence of 25 μM MG132, which was used to block proteasomal degradation. Interestingly, in absence of MG132, as Fig. 7 showed that, LMP1 increased the expression of p53 and the phosphorylation of p53, and in LMP1 negative cells, these proteins

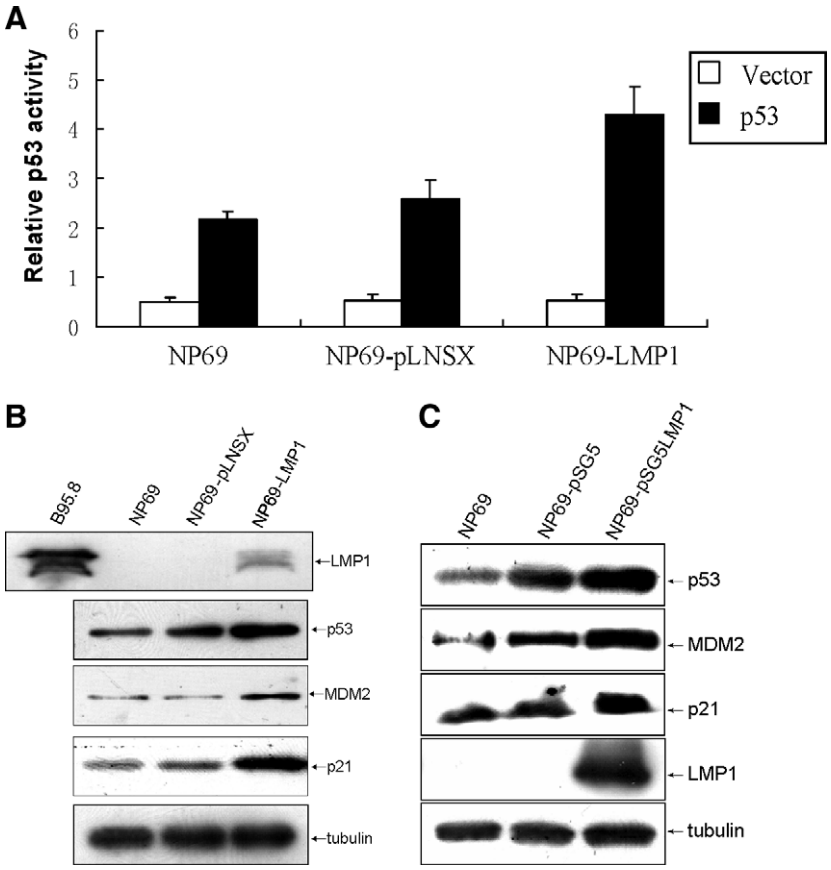

Fig. 6. The effects of LMP1 to p53 at transcriptional level and translational level. (A) p53 dependent transcription activity was measured by the luciferase assay, and results were expressed as relative p53 activity. β-Gal gene used as a control to normalize the variation in transfection efficiencies among each sample. Data from three independent experiments were averaged and are presented as mean ± S.E. (B) Effects of the expression of LMP1 on p53, MDM2 and p21 expression. (C) The plasmid pSG5-LMP1 was transiently transfected into NP69 cells. The pSG5 empty vector was also added as blank control. Immunoblotting analysis of p53, MDM2 and p21 in the transfected NP69 cells was shown. α-Tubulin protein level was detected as a loading control.

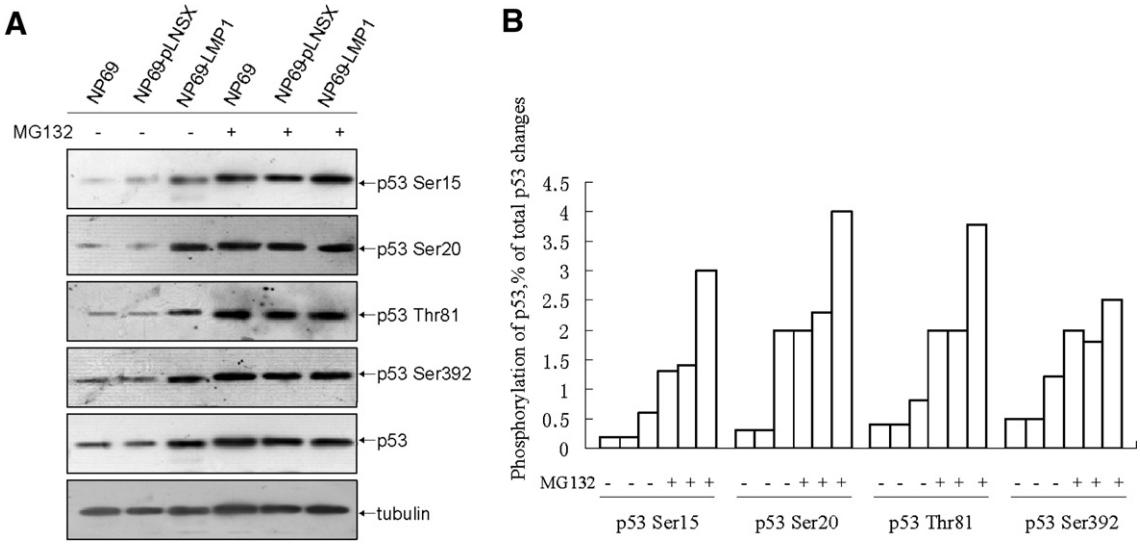

Fig. 7. The ubiquitination of p53 regulated by LMP1. (A) MG132 promotes the phosphorylation of p53 and accumulation. Induction of p53 expression and phosphorylation at serine 15, serine 20, serine 392 and threonine 81 in presence or absence MG132. Lysates were prepared from NP69, NP69-pLNSX and NP69-LMP1 cells, or untreated with the proteasome inhibitor MG132 (Calbiochem) for 6 h prior to harvesting. p53 phosphorylated on specific serine and threonine residues were analyzed by immunoblotting. α-Tubulin protein was as a loading control. (B) The relative amount of phosphorylated p53 was calculated as the percent of total p53 using densitometry.

showed little expression. But in the presence of MG132, the expression of p53 and phosphorylation of p53 increased obviously in LMP1 negative cells, as well as in LMP1 positive cells (Fig. 7). These data demonstrated that lots of p53 degraded through ubiquitin proteasome system in LMP1 negative NP cells, while, in LMP1 positive cells, LMP1 augmented the expression of p53 via inhibiting p53 proteolysis in 26 S proteasome. This is the first time to explain LMP1 regulated p53 through a post-ubiquitination mechanism.

Our previous studies also showed that LMP1 could induce the accumulation of p53 protein and upregulate its transcription activity in p53-mutant NP cells [30]. These results obtained here not only confirm the previously conclusion and reconcile the discrepant results obtained in the published studies cited earlier, but also reveal unanticipated complexities in the function of p53 in the carcinogenesis of NPC [31,32].

In conclusion, these findings for the first time elucidated the activation of p53 triggered by LMP1 in wild-type p53 NP cells. Another question that we need to consider is that p53 may has other function than inducing apoptosis, such as favor for virus replication and initiating the expression of oncogene, such as MDM2, in the carcinogenesis of NPC. The exploration of LMP1 regulated p53 will further define the role of p53 in the pathogenesis of NPC.

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