Investigating the influence of Epstein-Barr virus on the p53 pathway in nasopharyngeal carcinoma

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Abstract. Nasopharyngeal carcinoma (NPC) is a malignancy arising from the epithelial lining of nasopharynx. It is one of the most common malignancies in the southern area of China and South-East Asia. NPC is frequently reported in Sarawak, Malaysia especially in males and is endemic to certain ethnic groups, including the Bidayuh and Chinese. The development of NPC is associated with multiple factors, with one common mechanism involving persistent infection with Epstein-Barr virus (EBV). This study will aim to gain a clearer understanding regarding the mechanism of EBV influencing p53 pathway to facilitate NPC development. Mutations in p53 have been associated with oncogenesis of several human malignancies, however such mutations are relatively rare in NPC and the mechanisms through which p53 is inactivated in this malignancy remain poorly understood. In this study, we study the activity of p53 in immortalised nasopharyngeal epithelial cells using nutlin-3, an MDM2 antagonist. The impact of EBV infection on the protein expression of p53 and its ability to stimulate its transcriptional target genes is studied. The proteins levels of p53 and its transcriptional activation of target genes was significantly higher in EBV-infected cells, indicating that EBV infection alone is not sufficient to attenuate the p53 pathway in a non-malignant nasopharyngeal cell line. These observations will provide the fundamental steps towards the understanding of p53 and EBV viral proteins interaction that causes NPC.

1. Introduction

Nasopharyngeal carcinoma is a rare malignancy in other countries but it is endemic in southern part of China and South-East Asia which may be unique through the geographical distributions and genetic predispositions from common ancestor¹. The high incidences of NPC cases were also reported in Sarawak, Malaysia with male population at the highest risk compared to female population especially with the native ethnic group of Bidayuh². According to Malaysian National Cancer Registry report, NPC ranked fifth most common cancer in Malaysia with Sarawak at the highest among any other states³. In addition to the regional and racial distribution, NPC is commonly associated with EBV as almost all NPC cases reported to have EBV episomes⁴. EBV belongs to the gamma herpesvirus family with >90% of human population worldwide is infected. EBV infection has been observed in a number of human malignancies such as Burkitt’s lymphoma, Hodgkin’s lymphoma and gastric cancer. The infection is usually asymptomatic and only a small proportion of the infected individuals developed into NPC with almost all NPC cases recorded to be undifferentiated or poorly differentiated histological type⁵. EBV infection in human cancers is predominantly to be latent as different cancers manifest dissimilar latent
genes. The latent genes involves are three latent membrane proteins (1, 2A, 2B) and six EB nuclear antigens (1, 2A, 3A, 3B, 3C, LP). The latent genes commonly expressed in latency type II NPC were EBNA1, LMP1 and EBER with EBNA1 as the main gene for persistence production of EBV genomes. The tumour suppressor gene, p53 is frequently associated with human cancers because most of the human cancers have mutated p53. The p53 protein is commonly inactivated by viral proteins such as E6 of HPV. Although p53 mutations are prevalent in human cancers, p53 mutation is not common for NPC instead, p53 was elevated. The role and direct mechanism of p53 in NPC has yet to be fully understood. The study on the involvement of p53 and its transcriptional targets with EBV genes are of much interest.

2. Experimental Methods

Cell culture
Adherent cell lines were cultured in flasks maintained at 37°C in 5% humidified CO₂ in complete growth medium. Both NP460hTert and NP460hTert-EBV were human immortalised nasopharyngeal epithelial cell lines used for the experiment and maintained in a 1:1 ratio of complete Defined Keratinocyte-SFM (Gibco, Grand Island, NY) and Epilife® serum-free medium with Epilife™ defined growth supplement (EDGS) (Cascade Biologies, Portland, OR). The cells were passaged after reached confluency by trypsinising with 0.25% EDTA trypsin (Gibco, Grand Island, NY) for 5-10 minutes at 37°C. The cells were collected with RPMI 1640 supplemented with 2mM of L-glutamine (Gibco, Grand Island, NY) and 10% fetal bovine serum (FBS) and centrifuged at 200g for 5 minutes. The cell pellet was resuspended with fresh culture medium and seeded for subsequent experiments.

Proliferation assays
The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) based colorimetric assay (Merck Millipore, Billerica, MA) was used to determine the viability of NPC cells incubated with of nutlin-3 (Selleck Chemicals, Houston, TX). The assay was conducted as previously described with slight modifications. The cells were harvested and seeded in a 96-well plate at a density of 2x10⁵ cells/100µL in triplicates and were allowed to attach for 24 hours prior any treatment. Nutlin-3 was added to the 96-well plate in a two-fold dilution series at final concentrations ranging from 0.16 to 20µM. Vehicle controls (0.1% DMSO) also were prepared. Cell plates were incubated for 72 hours at 37°C and 20µL of MTT was added to each well after the incubation, followed by incubation for 4 hours at 37°C. The absorbance was measured at 490 nm using microplate reader (Tecan, Mannedorf, Switzerland).

Real-time quantitative PCR (RT-qPCR)
The total RNA was extracted from cells using RNeasy Mini kit (Qiagen, Hilden, Germany). The first-strand cDNA synthesis was conducted using High Capacity cDNA reverse transcription kit (Applied Biosystems, CA, US). The relative gene expression changes were determined by qPCR on cDNA using QuantiTerra SYBR Green PCR kit (Qiagen, Hilden, Germany). The qPCR reaction mixture which consists of 20 µL volume was prepared in a qPCR 96-well plate according to manufacturer’s instruction. The primers used for the experiment were purchased from Helix Biotech, Selangor, Malaysia. Primers used for the experiment were Bax (forward: 5'-ACGAACTGGACAGTACATGGAG-3' and reverse: 5'-CAGTTTTGCTGGCAAAAGTGGAAAG), GAPDH (forward: 5'GAAGGTGAAGGTCGGAGTC-3' and reverse: 5'-GAAGATGGTGATGGTGATCATGTC-3'), MDM2 (forward: 5'-TCTACAGGGACGCCATCGA-3' and reverse: 5'-TCTAGTCAACAATCACTCTA-3'), p21 (forward: 5'-TTAGGCTTCTCTTCTGGAGATTC-3' and reverse: 5'-TTAGGCTTCTCTTCTGGAGATTC-3') and p53 (forward: 5'-CTTCATGCATATCGAAGGAGATGC-3' and reverse: 5'-TTAGGCTTCTCTTCTGGAGATTC-3'). The assay was performed using Applied Biosystems 7500 Fast Real-time PCR system and analysed with 7500 fast system SDS software v2.0.5 The qPCR cycling parameters were 2 mins at 95°C for hot start, followed by 35 cycles of denaturation at 95°C for 5 secs, combined annealing and extension at 60°C for 30 secs. The melting curve analysis was verified to ensure that the amplified PCR product from each
primer pair produces one specific product. The relative quantification of gene expression was done using comparative ΔΔCt method with normalization of target gene to the control gene, GAPDH.

Immunoblot analysis
Protein quantification was carried out using Bradford assay\textsuperscript{16}. Briefly, the cell pellet was lysed with RIPA lysis buffer supplemented with protease and phosphatase inhibitors, followed by sonication and incubation on ice before centrifuged to pellet the debris. The supernatant was collected, followed by addition with Bradford reagent and quantified using a bovine serum albumin (BSA) standard curve (Bio-Rad, CA, US). The absorbance was subsequently measured at 595 nm. Protein analysis was conducted as previously described\textsuperscript{17}. Protein samples (20 µg) obtained was resolved on SDS-PAGE. After electrophoresis, the polyacrylamine gels containing resolved proteins were transferred to a PVDF membrane (Amersham, Buckinghamshire, UK). The membrane was blocked using 5 % skim milk in TBS-T for 1 hr at room temperature with shaking. The membrane was then incubated with beta-actin or p53 primary monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:3000 and 1:1000 dilution respectively for overnight at 4°C. After overnight incubation, the membrane was incubated with anti-mouse secondary antibodies conjugated with HRP (Santa Cruz Biotechnology, Santa Cruz, CA) at dilution of 1:5000 at room temperature for 1 hr. The membrane was then rinsed with TBS-T and the protein of interest was developed using chemiluminescent (ECL) detection system (Advansta, Menlo Park, CA) and visualised under Odyssey Imaging Systems (Li-COR Biosciences, Nebraska, US).

3. Experimental Results
In this study, we initially investigated whether EBV infection of a non-malignant nasopharyngeal epithelial cell line (NP460hTert) could attenuate the p53 pathway. To study the cell’s response to p53 pathway activation, NP460hTert or NP460hTert-EBV cells were incubated with various concentrations of nutlin-3 and the viability of both cell lines was determined. Both cell lines were sensitive to nutlin-3 treatment and showed dose-dependent cytotoxic responses. The average inhibition concentration (IC\textsubscript{50}) obtained for NP460hTert and NP460hTert-EBV cells were 9.4 µM ± 0.37 and 4.15 µM ± 0.89 respectively (Figure 1). From the results, the treatment with nutlin-3 had a more significant effect on NP460-hTert-EBV cells as compared to NP460 cells (Student t-test, \(p<0.05\)). The IC\textsubscript{50} of EBV-infected cells was twice more than non-EBV cells showing that EBV-infected cells are more sensitive towards nutlin-3.

![NP460hTert cells induced with nutlin-3](image1)

**Figure 1.** Cell viability curve induced with nutlin-3 at 72 hours
The impact of EBV on the ability of p53 to function as a transcription factor was subsequently explored. NP460hTert and NP460hTert-EBV cells were incubated with nutlin-3 (10μM) or vehicle control for 24 hours, and the expression of p21, MDM2 and Bax genes were analysed. Bax was shown to be transcriptionally induced 3 – 4-fold by p53 in NP460hTert-EBV cells, however its expression was unaltered upon nutlin-3 treatment in NP460hTert cells (Figure 2). Similarly, MDM2 and p21 mRNA levels were highly expressed in NP460hTert-EBV cells, with both MDM2 and p21 immensely expressed with approximately 50 and 160-fold respectively (Figure 2). Interestingly, the ability of nutlin-3 to induce the expression of these p53 target genes was significantly lower in NP460hTert cells. The p53 transcriptional targets did not show any significant changes following nutlin-3 treatment of NP460hTert cells. In contrast, p21, MDM2 and Bax genes for NP460hTert-EBV induced with nutlin-3 were significantly expressed (Student’s t-test, p<0.0001, p<0.0001 and p<0.001 respectively). We also found that p53 mRNA level were significantly lower expressed (approximately 2-fold) in NP460hTert-EBV cells as compared to NP460hTert cells after induced with 10 μM of nutlin-3 for 24 hours (Figure 2).
Figure 2. Gene expression of p53 transcriptional targets

Protein analysis was also conducted to determine the impact of EBV on p53 protein levels. The basal level of p53 proteins was approximately 3-fold higher in NP460hTert cells as compared to NP460hTert-EBV cells (Figure 3) as determined by densitometry analysis. The p53 protein levels for both nutlin-3 induced cells were significantly higher as compared to its basal level but NP460hTert-EBV had the highest induction of p53 protein levels with approximately six-fold more than basal levels.

Figure 3. Protein expression of p53

4. Discussion

In this study, the stability and activation of p53 and its transcriptional targets is crucial for the determination of progression or aversion of cancer malignancies\(^\text{18}\). The important protein involves in the regulation of p53 is MDM2, whereby p53 level is controlled by MDM2 under non-stressed condition. The interaction between p53 and MDM2 causes p53 to be ubiquitinated and eventually degraded, hence by blocking the interaction allows p53 to be activated if stresses are detected\(^\text{19,20}\). One of the commonly used drugs developed for such affinity is nutlin-3 which is widely used in cancer studies. The activation of p53 pathway has been outlined to induce cell cycle arrest or apoptosis through its transcriptional targets such as p21 and Bax\(^\text{21}\). This indicates that not all mRNAs are equally translated into proteins because the regulation of transcription and translation varies at different conditions\(^\text{22}\).

The results indicates that NP460hTert-EBV cells retained its wild-type (wt) p53 as nutlin-3 is cytotoxic only to cells with wt-p53 as suggested by previous studies\(^\text{17,23}\). This also shows that there is abundance of wt-p53 in the EBV-infected cells and EBV might contribute towards the accumulation of wt-p53 compared to normal cells. The cells without induced with nutlin-3 had higher p21 levels than p53 levels which happened under normal condition and not under drug-induced DNA damage. The consistency in higher p21 levels were seen under normal and treated NP460hTert-EBV cells in Figure 2 suggest that p21 transactivation is independent of p53 and possibly represses p53 at certain level. However, MDM2 was supposed to be inhibited from binding with p53 when treated with nutlin-3 hence p53 levels are more likely to increase because it is not degraded but instead, p53 levels were still low.
The highly upregulated p21 mRNA levels might suggest that p21 is a negative regulator of p53 as studies showed that p53 decreases when p21 levels were highly expressed\textsuperscript{24}. In this situation, p53 in NP460 cells might be dependent on p21 expression whereby as described, p21 inhibits the induction of p53 even though p21 is the downstream transcriptional target for p53 activation involves in growth arrest\textsuperscript{25}. Although the presence of nutlin-3 inhibited the binding of MDM2 to p53, MDM2 was not readily degraded by proteolysis due to its level of abundance as shown in Figure 2, p53 levels remained low although induced with nutlin-3 as cells might readily undergo cellular repair as indicated by high levels of p21. Whereby, the apoptotic process is largely controlled by the Bcl-2 family group which includes Bax and Bcl-2 proteins. Bax is known to have a p53-binding domain which binds in the presence of p53 hence stimulating apoptosis\textsuperscript{26}. The presence of p21 might be one of the factors causing Bax to be locally expressed due to cells favouring cellular repair instead of undergoing apoptosis as damaged incurred by nutlin-3 was not severely cytotoxic.

In contrast, p53 protein level in NP460hTert-EBV cells was highly expressed after induction with nutlin-3. This showed that p53 is abundant in EBV-infected cells and EBV does not interfere with the expression of p53 proteins. The results also suggested that p53 protein might be in its wild-type condition as nutlin-3 is cytotoxic to cells with wt-p53. It is widely accepted that p53 is controlled by its stability where wt-p53 has shorter half-lives\textsuperscript{27}. There is evidence showing that p53 protein half-lives are higher in tumour cells\textsuperscript{28}. This might explain that EBV-infected has possible oncogenic properties but it does not induce cells into cancer cells. The results also suggest that p53 protein half-lives are likely to be higher as compared to mRNA level due to the rapid needs of p53 activation after induced by nutlin-3. One study showed that unstable mRNAs and proteins has enhanced transcription factors, gene signaling and chromatin modifications hence these regulatory genes were considered to have shorter half-lives\textsuperscript{29}. The immediate translational process of mRNAs could be the reasons for low p53 mRNAs detected as cells were stressed with nutlin-3 therefore producing more p53 protein for cellular repair/senescence. This gives an insight to understand the complex relationships between variable genes involves in normal and EBV cells thus better approach to look in development of EBV and NPC.

5. Conclusion

In conclusion, nutlin-3 enhanced the response of EBV-infected cells. Further studies are required to understand the complicated molecular mechanisms involving cellular transcriptional targets and EBV genes. This could be an important approach as to better understand the molecular pathogenesis of NPC. The findings will provide the fundamental steps towards future development of therapeutic strategies based on viral proteins that cause development of NPC.

Acknowledgement

This project was funded by the Ministry of Higher Education (MoHE) Fundamental Research Grant Scheme (#154671). We thank Professor George Sai Wah Tsao for providing the NP460hTert and NP460hTert-EBV cells, and for Dr Yap Lee Fah for her useful discussions.

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