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HDM2 impairs Noxa transcription and affects apoptotic cell death in a p53/p73-dependent manner in neuroblastoma

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ABSTRACT

HDM2, a human homologue of MDM2, is a major negative regulator of p53 function, and increased expression of HDM2 by its promoter polymorphism SNP309 resulted in p53 inactivation and an increased risk of several tumours, including neuroblastoma (NB). Herein, we show that increased expression of HDM2 is related to a worse prognosis in MYCN-amplified NB patients. HDM2 plays an important role in the expression of Noxa, a pro-apoptotic molecule of the Bcl-2 family, which induces NB cell apoptotic death after doxorubcin (Doxo) treatment. Knockdown of HDM2 by siRNA resulted in the upregulation of Noxa at mRNA/protein levels and improved the sensitivity of Doxo-resistant NB cells, although these were not observed in p53-mutant NB cells. Noxa-knockdown abolished the recovered Doxo-induced cell death by HDM2 reduction. Intriguingly, resistance to Doxo was up-regulated by over-expression of HDM2 in Doxo-sensitive NB cells. By HDM2 expression, p53 was inactivated but its degradation was not accelerated, suggesting that p53 was degraded in a proteasome-independent manner in NB cells; downstream effectors of p53, $p21^{Cip1/Waf1}$ and Noxa were suppressed by HDM2. Noxa transcription was considerably regulated by both p53 and p73 in NB cells. Furthermore, *in vivo* binding of p53 and p73 to Noxa promoter was suppressed and Noxa promoter activation was inhibited by HDM2. Taken together, our results may indicate that the HDM2-related resistance to chemotherapeutic drugs of NB is regulated by p53/p73-dependent Noxa expression in NB.

1. Introduction

Neuroblastoma (NB) is the most common extracranial solid malignant tumour in childhood, and derives from the sympathetic nervous system. Although the survival of many childhood malignancies has been improved by recent therapies, high-risk NB is still one of the most difficult tumours to cure, with only 30% long-term survival despite intensive

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multi-modal therapy and new treatments, and better understanding of drug resistance mechanisms is required.[1,2]

A noteworthy finding of NB research is that mutations of the p53 tumour suppressor have been reported in less than 2% of the 340 NBs tested.[3] Instead of p53 mutation, several molecular mechanisms have been proposed as alternatives of the inactivation in NB cells. Previously, p53 sequestration in cytoplasm was reported in primary tumour samples using immunohistochemical techniques,[4] however, several groups, including ours, have reported nuclear p53 accumulation in NB cells harbouring wild-type p53 after DNA damage,[5–8] and upregulation of the p53 downstream genes encoding $p21^{Cip1/Waf1}$ and HDM2 in p53 wild-type NB cell lines was observed in several studies.[3,7–10]

Although the molecular mechanisms of p53 inactivation in NB have not been completely elucidated, the role of MDM2 human homologue HDM2 has been a focus in the inactivation not only of NB but also of many other cells. HDM2 is an E3 ubiquitin ligase for p53 and retinoblastoma protein, and targets tumour suppressors for degradation by the ubiquitin-proteasome pathway.[11,12] This protein-protein interaction also blocks the p53 trans-activation domain and thereby inhibits p53 transcriptional activity.[13] Importantly, in certain situations, the over-expression of HDM2 results in p53-HDM2 complex that fails to bind tightly to DNA and thus causes the subsequent inhibition of p53 activity.[14] Furthermore, many cancer cell lines have high levels of oncogenic HDM2 proteins because of single nucleotide polymorphism (SNP) at position 309 in the first intron of the HDM2 gene; the p53 pathway is compromised in cells homozygous for HDM2 SNP309.[15]

At present, HDM2 functional analysis in NB cells has been performed by several laboratories. First, non-syntenic amplification of *HDM2* and *MYCN* and subsequent abundant HDM2 protein were reported in NB tumour samples and cell lines.[16,17] Second, HDM2 is involved in p53 degradation in NB cells and its activity is affected by Doxo treatment.[9] Third, intriguingly, MYCN directly binds to HDM2 promoter and regulates its transcription.[18] Fourth, it should be noted that small-molecule HDM2 inhibitor Nutlin-3 activates p53 and its downstream effectors and induces apoptotic cell death in NB cells although the effector of HDM2-regulated cell death in the p53 pathway remains to be elucidated.[19,20] Furthermore, HDM2 SNP309, a single nucleotide polymorphism in HDM2 promoter-enhancing HDM2 expression, in NB tumours is related to NB development and aggressiveness, especially in the advanced-stage and MYCN-amplified tumours.[21,22]

Previously, in HDM2 highly expressed NB cells, we found that p53 was functionally inactivated and activation of the p53 downstream effector Noxa was attenuated in some NB cells,[8] suggesting that HDM2 has a role in p53 functional inactivation in some NB cells harbouring wild-type p53. Importantly, our findings are consistent with previous findings that the impairment of HDM2-dependent p53 degradation was observed in cancer cells.[23] These results led us to study the role of HDM2 in the molecular machinery of p53-dependent cell death, especially in Noxa regulation in NB. Consequently, the crucial role of the HDM2/p53/p73 pathway in apoptosis and Noxa expression in NB cells was indicated by our observations.

2. Materials and methods

2.1. Tumour samples

Fresh, frozen tumour tissues were sent to the Division of Biochemistry, Chiba Cancer Center Research Institute, from various hospitals in Japan with informed consent from the patients' parents. All samples were obtained by surgery or biopsy and stored at −80 °C. More than 70% tumour cell content of the samples was confirmed by pathological analysis of the adjacent tissues. Ninety two *MYCN*-single copy NB and 26 *MYCN*-amplified NB samples were studied. Studies were approved by the Institutional Review Board of Chiba Cancer Center.

2.2. Reagents and antibodies

Anti-p53 mouse monoclonal antibody (clone DO-1), anti-$p21^{Cip1/Waf1}$ mouse monoclonal antibody (clone F-5) and anti-HDM2 mouse monoclonal antibody (clone SMP14) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-p53 rabbit serum (p53ser15p, p53ser20p and p53ser46p) and anti-phospho-p53ser15 mouse monoclonal antibody (clone 16G8) were from Cell Signaling Technology (Beverly, MA). Anti-p53 mouse monoclonal antibody (clone pAb421) and anti-HDM2 mouse monoclonal antibody (clone 2A10) were from Oncogene Research Products (Cambridge, MA). Anti-Noxa monoclonal antibody (clone 114C307) was from Abcam (Cambridge, UK). Anti-actin serum and anti-FLAG M2 monoclonal antibody were from Sigma–Aldrich (St Louis, MO). Nutlin-3 was from Alexis Biochemicals (Plymouth Meeting, PA, USA). Other biochemical reagents were purchased from Sigma–Aldrich Japan or Wako (Osaka, Japan).

2.3. Cells and cell culture

We obtained p53 wild-type NB cell lines to study the role of the p53 pathway in the drug resistance mechanism of NB cells from several cell banks, and the p53 and MYCN statuses were studied as previously described.[8] Briefly, p53 wild-type NB cell lines are SK-N-SH, NB-9, IMR32 and NB-19; p53-mutant NB lines are SK-N-DZ and SK-N-BE. MYCN status is single copy in SK-N-SH and amplified in NB-9, IMR32, NB-19, SK-N-BE and SK-N-DZ. For luciferase assay, we used H1299 cells because of high transfection efficiency.[24] The cells were routinely maintained with DMEM supplemented with 10% foetal bovine serum (FBS) and 1 × penicillin/streptomycin (Gibco BRL). Cell proliferation was assessed by cell number counting and the WST (WST: water-soluble tetrazolium salt) assay according to the manufacturer's protocol (Cell Counting Kit-8; Dojindo, Kumamoto, Japan).

2.4. Morphological analysis of apoptosis and analysis of sub-G0/G1 fraction

Cells were observed using a phase-contrast microscope to assess apoptotic morphological changes, and treated with DAPI, a DNA-staining dye, to detect the morphological characteristics of apoptotic nuclei, namely, condensation and fragmentation, after fixation with 3.7% (v/v) formaldehyde/1 × PBS.

The sub-G0/G1 fraction was analysed using the method described in the previous report.[8]

2.5. Immunoprecipitation and direct Western blotting

Immunoprecipitation and Western blotting were performed basically according to previous reports.[25,26] For the detection of p53 after immunoprecipitation, goat anti-mouse kappa (κ chain-specific) antiserum conjugated with horseradish peroxidase (SouthernBiotech, Birmingham, AL, USA) was used to reduce the background.

2.6. Preparation of mRNA and analysis of RNA expression

Total RNA was extracted from NB cells using Isogen® (Wako, Tokyo, Japan), and cDNA was synthesised from 1 μg total RNA templates according to the manufacturer's protocol (RiverTra-Ace-α-RT-PCR kit; Toyobo, Osaka, Japan). Semi-quantitative PCR amplification and quantitative real-time PCR were performed using previously reported primers.[24] Primer sequences are shown in Supplementary Table S1.

2.7. Retrovirus production and infection

HDM2 cDNA was cloned into pSR-alpha-MSV tkNeo vector; retrovirus production, infection and confirmation of infection efficiency were performed as described previously.[24]

2.8. Small interfering RNA transfection

HDM2 and Noxa small interfering RNAs were synthesised according to previous papers.[8,12] Control siRNA (Silencer® Negative Control #1 siRNA) was purchased from Ambion Inc. (Austin, TX, USA). p53 siRNA (ON-TARGETplus Duplex J-003329-14-0005, Human Tp53) was from Thermo Fisher Scientific (Lafayette, CO, USA). p73 siRNA (SignalSilenceR p73 siRNA) was from Cell Signaling Technology Inc. (Danvers, MA, USA). NB cells were plated at a density of 3×10^5 cells in a 3 cm diameter dish treated with 10 nM small interfering RNA duplexes/Lipofectamine™ RNAiMAX complexes according to the manufacturer's protocol (reverse transfection; Invitrogen, Carlsbad, CA, USA). On the following day, 5 nM small interfering RNA duplexes were transfected with Lipofectamine™ RNAiMAX in Opti-MEM medium according to the manufacturer's protocol (forward transfection). Twenty-four hours after forward transfection, the cells were subjected to analysis or treated with Doxo. To check the effect of siRNAs on the stimulation of innate immune responses and suppression of off-targets, we analysed *Interferon-alpha1*, *Interferon-beta*, *Bax*, *Bak* and $p21^{Cip1/Waf1}$ mRNA expression by semi-quantitative RT-PCR as described previously[8] and found an almost identical expression between mock- and interest gene-siRNA-treated cell samples (data not shown).

2.9. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as previously described.[27,28] To check the effect of HDM2 on p73 binding to Noxa promoter, pCDNA3-FLAGp73 was transfected into NB cells before ChIP assay. SK-N-SH and SK1 cells were seeded 24 h before transfection at a density of 1.5×10^6 in a 10 cm dish. The cells were transfected with pCDNA3-FLAGp73/Lipofectamine 2000 in Opti-MEM medium according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Doxo treatment was performed 48 h after transfection. Cross-linked chromatin prepared from the indicated cells was precipitated with normal mouse IgG (eBioscience), monoclonal anti-p53 antibody (DO-1) or monoclonal anti-FLAG M2 antibody; ChIP assay and real-time PCR were performed.

2.10. Luciferase reporter assay

The −423/+413 and +95/+413 5′-upstream fragments (5′-end of Noxa exon 1 was determined as +1 position) were subcloned into luciferase reporter plasmid pGL3-basic Luciferase Reporter Vector (Promega, Madison, WI, USA). The putative p53-binding site is −184/−175.

H1299, SK-N-SH and SK-N-BE cells were seeded in a 12-well plate 24 h before transfection at a concentration of 5×10^4 cells/well. The cells were co-transfected with Renilla luciferase reporter plasmid (pRL-TK), pCDNA3-p53 or pCDNA3-p73, pCMV-HDM2 and luciferase reporter plasmid with the above-mentioned Noxa promoter fragments. The total amount of plasmid DNA per transfection was kept constant (510 ng) with pBlueScript KT+. Forty-eight hours after transfection by Lipofectamine 2000, the cells were lysed and their luciferase activities were measured by the Dual-Luciferase reporter system (Promega, Madison, WI, USA). The firefly luminescence signal was normalised based on the Renilla luminescence signal.

3. Results

3.1. Expression of HDM2 in primary NB tumours

First, we studied *HDM2* mRNA expression in 116 primary NB tumours and normalised using *GAPDH* values by quantitative real-time PCR as described in Methods. We then examined whether there could exist a possible correlation between the expression levels of HDM2 in primary NB and the survival of patients with NB by Kaplan–Meier analysis (Fig. 1). We could not see a difference between the HDM2 low expression group ($n = 46$) and high expression group ($n = 46$) of MYCN-single copy NB in patient prognosis ($n = 92$). However, the log-rank test showed a correlation between the higher expression of HDM2 and an unfavourable outcome ($p < 0.05$) in MYCN-amplified patients ($n = 26$). In MYCN-amplified patients, 6 of 13 patients were alive in the HDM2 low expression group although only 1 of 13 patients was alive after more than 5 years of observation ($p < 0.005$, by Fisher's exact probability test). We evaluated whether there could exist a possible relationship between HDM2 levels and clinicopathological factors of NB patients (Supplementary Tables S2 and S3) and found that there was no significant difference between the HDM2-high and HDM2-low groups with respect to age, tumour origin, INSS stage, Shimada's pathological classification and DNA index. Together, our data suggest that HDM2 expression has some impact on the prognosis of MYCN-amplified NB patients and we studied the mechanisms of the HDM2-related aggressiveness of NB.

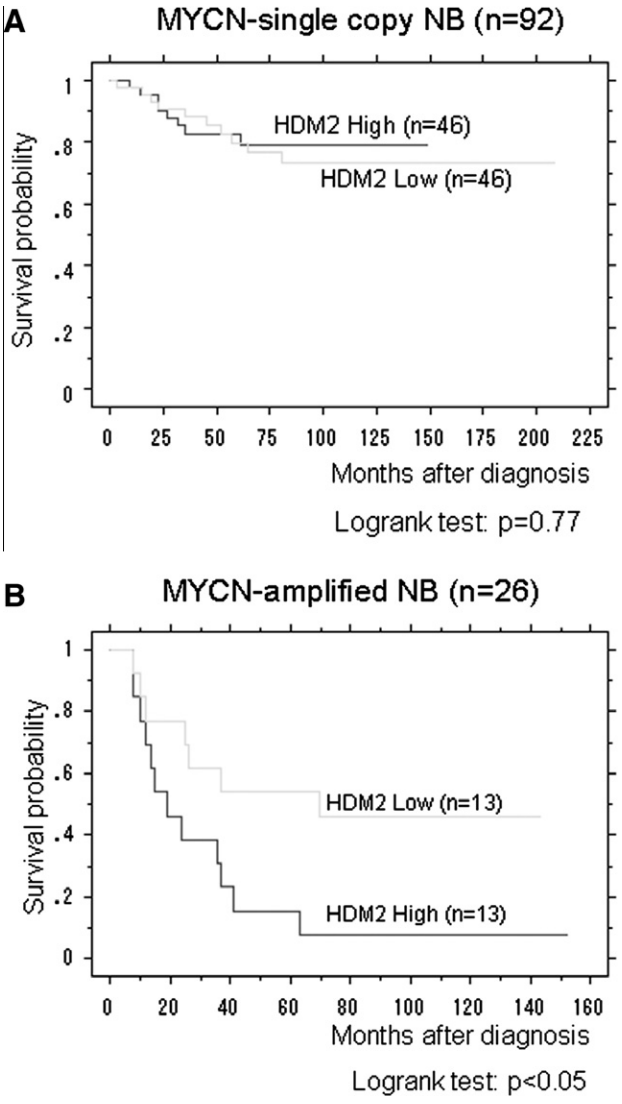

Fig. 1 – HDM2 higher expression correlates with unfavourable prognosis in MYCN-amplified NB patients. Kaplan–Meier survival curves of NB patients based on expression levels of *HDM2*. Expression levels of *HDM2* in 92 MYCN-single copy NB samples (A) and in 26 MYCN-amplified NB samples (B). Relative expression levels of *HDM2* were determined by normalisation of *HDM2* mRNA amounts by *GAPDH* mRNA.

3.2. *HDM2 is highly expressed in Doxo-resistant NB cells*

In the previous paper,[8] we identified SK-N-SH and NB-9 as Doxo-sensitive cells and IMR32 and NB-19 as Doxo-resistant cells. We have confirmed the resistance of IMR32 and NB-19 to Doxo by WST-8 assay in the present report (Fig. 2A). Both p53 and HDM2 proteins were accumulated in Doxo-resistant cells (Fig. 2B) and this HDM2 accumulation was caused at the transcriptional level (Fig. 2C). Co-localisation of p53/HDM2 was observed in the nucleus (data not shown)[8] and a clear interaction of these endogenous proteins was detected by immunoprecipitation-western blotting in those cells (Supplementary Fig. S1), suggesting that p53 may be inactivated not by protein degradation but by a physical interaction with HDM2 in NB cells. To confirm this, we treated SK-N-SH, NB-19, IMR32 and HDM2 over-expressing SK-N-SH cells (SK1) with a proteasome inhibitor MG132 and found that p53 accumulation was not observed in NB-19, IMR32 and HDM2 over-expressing SK1 cells (Fig. 2D), indicating that proteasome-dependent degradation of p53 was impaired in these cells.

3.3. *HDM2 knockdown sensitises resistant NB cells to Doxo treatment*

Accumulation of HDM2 in NB cells led us to consider that p53-related cell death pathways may be inactivated by HDM2. To confirm this, we studied the effects of HDM2 knockdown in Doxo-treated NB cell lines. Although Doxo- or HDM2 siRNA-single treatment was not so effective, Doxo/HDM2 siRNA-combined treatment successfully induced morphological changes in cell death (shrunken and floating cells) and trypan blue uptake in p53 wild-type IMR32 and NB-19 cells, but not in p53-mutated SK-N-DZ cells (Supplementary Fig. S2). Nuclear condensation and fragmentation indicated that this cell death was caused by apoptosis induction (Fig. 3A and B). We studied the expression of the pro-apoptotic Bcl-2 family molecule, Noxa, in Doxo- and/or HDM2 siRNA-treated NB cells because we found that Noxa is a critical molecule in p53-related damage-induced NB cell death.[8] In both IMR32 and NB-19 cells, HDM2 knockdown induced Noxa both at mRNA and protein levels (Fig. 3C and D). Meanwhile, an additive effect of Doxo and HDM2 siRNA treatment was not observed in p53-mutated SK-N-DZ cells, suggesting that HDM2 regulates Noxa transcription after Doxo treatment in a p53-dependent manner.

Next, we confirmed the significance of Noxa in HDM2-related cell death in Doxo-resistant NB cells. HDM2 single-knockdown and HDM2/Noxa double-knockdown effectively decreased *HDM2* and/or *Noxa* mRNAs (Fig. 3D). HDM2 single-knockdown significantly induced cell death caused by Doxo treatment (Fig. 3E). It was noteworthy that Noxa-knockdown effectively abolished Doxo-induced cell death increased by HDM2 single-knockdown in IMR32 and NB-19 cells.

We also studied the effect of the small-molecule inhibitor of HDM2 Nutlin-3 on Doxo-resistant NB cells because the effectiveness and inhibitory mechanism of this molecule have been studied substantially.[29] These experiments confirmed that HDM2 regulates the transcription of Noxa and apoptotic cell death caused by Doxo treatment (Supplementary Fig. S3). Taken together, Noxa plays an indispensible role in HDM2-regulated cell death after Doxo treatment in NB.

3.4. *HDM2 over-expression confers drug resistance and inactivation of p53 on Doxo-sensitive NB cells*

To address the role of HDM2 in NB apoptotic cell death, we introduced HDM2 into Doxo-sensitive SK-N-SH cells using a retrovirus vector and isolated several clones (SK1, SK2 and SK3) by G418 selection. HDM2 expression of the representative clones is shown in Fig. 4A. Intriguingly, the percentages

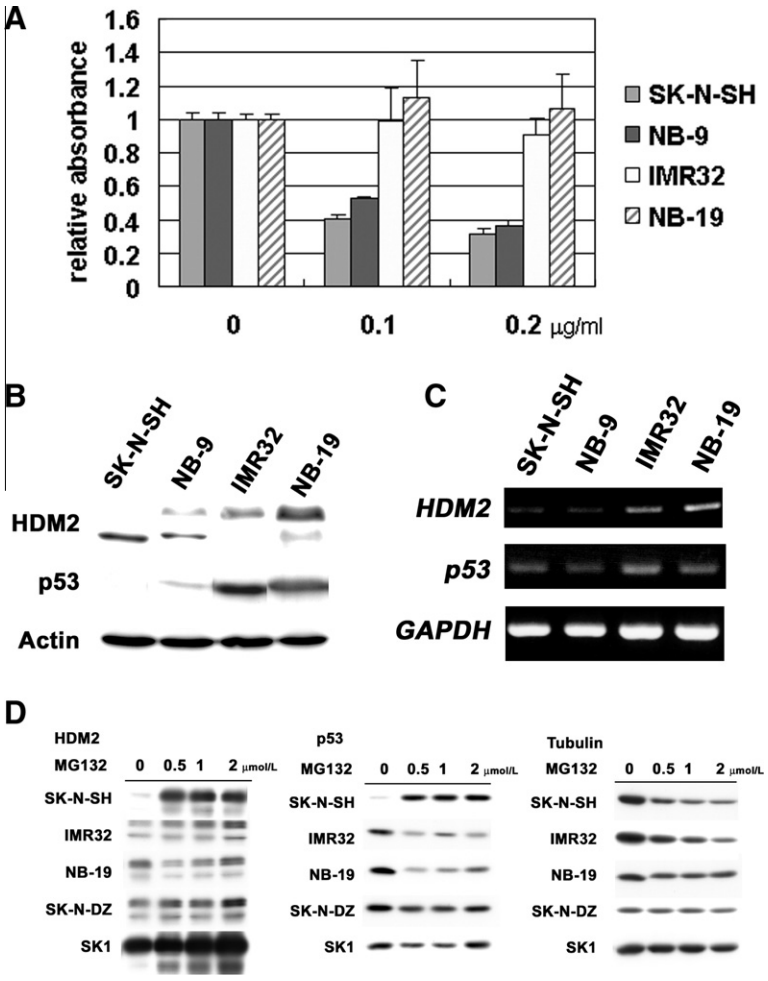

Fig. 2 – HDM2 is considerably expressed in Doxo-resistant NB cells. (A) p53 wild-type IMR32 and NB-19 cells are Doxo-resistant. 10^4 Cells were seeded in 96-well plates and treated with the indicated concentration of Doxo. WST assay was performed. The results are representative of three independent experiments. (B) HDM2/p53 protein expression in NB cells. Total protein was extracted and subjected to Western blotting analysis with anti-HDM2 2A10, anti-p53 DO-1 or anti-Noxa antibody. (C) *HDM2/p53* mRNA expression in NB cells. Total RNA extraction, cDNA synthesis and semi-quantitative RT-PCR were performed. (D) Indicated cells were treated with 2 μM MG132 for 24 h and lysed. Protein extracted from cells was subjected to SDS–PAGE and western blot analysis. HDM2-overexpressing SK-N-SH cell was 'SK1' in Figs. 4, 6 and 7.

of trypan blue uptake cells and the sub-G0/G1 fraction were significantly decreased in HDM2-overexpressing cells (Fig. 4B and C). Furthermore, cell proliferation was accelerated in HDM2-overexpressing cells although proliferation speeds were considerably different among the clones (Fig. 4D).

Next, we studied the amount of p53 and its phosphorylation status after Doxo treatment in HDM2-overexpressing clones. Accumulation of p53 without Doxo treatment and phosphorylation inhibition were observed in HDM2-overexpressing clones (Fig. 4E). Mutation analysis of p53 of these clones was performed and no p53 mutation was found, and HDM2/p53 interaction was confirmed by immunoprecipitation-western blotting (data not shown). Induction of $p21^{Cip1/Waf1}$ and Noxa was not detected in these clones after Doxo treatment (Fig. 4E). Taken together, p53 was inactivated by HDM2 over-expression in Doxo-sensitive SK-N-SH cells and Noxa expression was profoundly suppressed in these cells, confirming the significance of HDM2 in p53/Noxa pathway regulation.

3.5. *Noxa transcription in steady state is regulated by both p53 and p73 in NB cells*

Next, since *Noxa* mRNA was considerably detected in p53 functionally inactivated NB cells (IMR32 and NB-19),[8] we studied *p53*, *p73* and *Noxa* mRNA expression in several NB cell lines which have a different p53 status (Fig. 5A). Noxa expression was remarkably decreased in p53-mutated cell lines, even in p73-expressing SK-N-DZ cells; however, Noxa expression was observed in 3 of 4 p73 highly expressed NB cell lines (SH-SY5Y, NGP and IMR32). These results prompted us to knock down p53 or p73 and to assess their roles in *Noxa* mRNA expression (Fig. 5B). In IMR32 cells, both p53 and p73 knockdown effectively reduced *Noxa* mRNA; p53/p73 double-

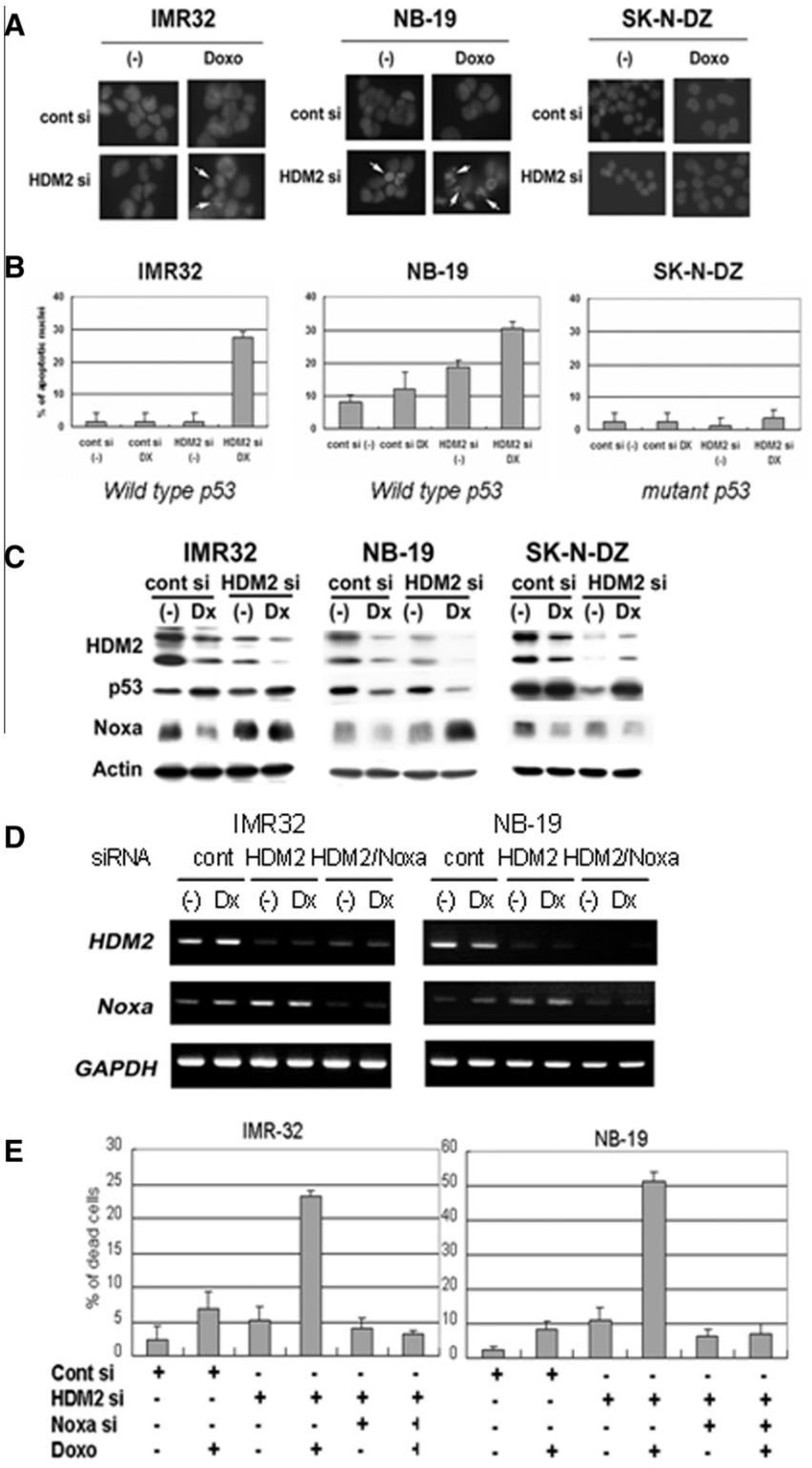

Fig. 3 – HDM2 knockdown attenuates Doxo-induced apoptotic cell death in NB cells. NB cells were transfected with siRNAs. Twenty-four hours after the 2nd siRNA transfection by forward transfection, the cells were treated with 0.3 μg/ml Doxo. The results are representative of three independent experiments. (A and B) DAPI staining of NB cells. Twenty-four hours after Doxo treatment, the cells were stained with DAPI and observed using a confocal laser-scanning microscope (A, white arrow: fragmented nuclei) and fragmented nuclei were counted. The percentage of fragmented nuclei is presented as bar graphs (B). (C) Expression of HDM2/p53/Noxa proteins. Total protein was extracted 36 hours after Doxo treatment, and then subjected to Western blotting with anti-HDM2 2A10, anti-p53 DO-1 or anti-Noxa antibody. (D) Expression of *HDM2/Noxa* mRNA. Total RNA extraction, cDNA synthesis and semi-quantitative RT-PCR were performed as described in Methods. (E)Trypan blue analysis of NB cells. Twenty-four hours after Doxo treatment, the cells were analysed.

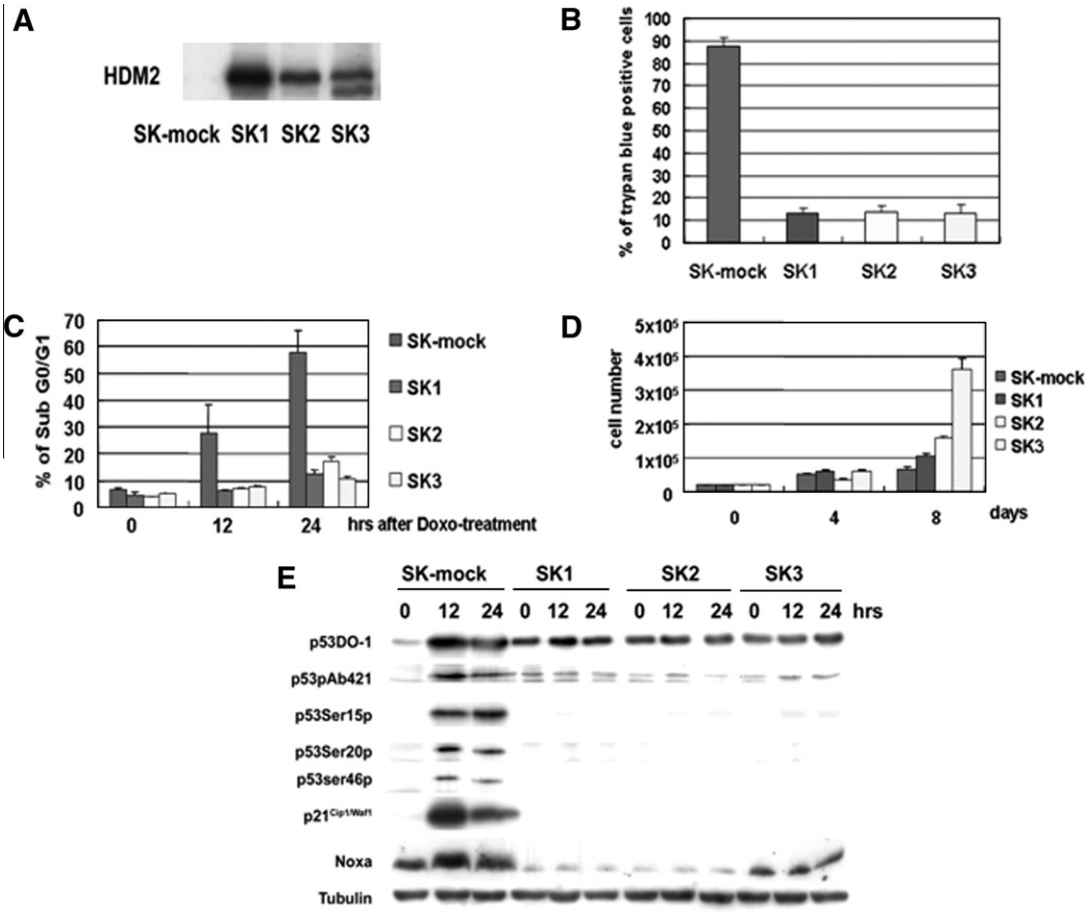

Fig. 4 – HDM2 over-expression in Doxo-sensitive NB cells mimics Doxo-resistant phenotype. The results are representative of three independent experiments. (A) HDM2 was introduced by retrovirus vector. HDM2 was detected by Western blotting with anti-HDM2 2A10 antibody. (B and C) One hundred thousand cells were plated in a 3 cm diameter culture dish and cultured in 5% CO_2 for 24 h. Doxo was added to the dish at 0.3 μg/ml and then incubated for 24 h. In Fig. 4B, trypan blue uptake assay was performed 24 h after Doxo treatment. In Fig. 4C, the sub-G0/G1 fraction was analysed at the indicated time points. (D) Twenty thousand cells were plated in a 3 cm diameter culture dish and cultured in 5% CO_2, and the cell number per dish was counted at the indicated time points. Mean and standard deviation of the % of cells were calculated for triplicate samples. Results are representative of three independent experiments. (E) Total cell lysates were subjected to SDS–PAGE and Western blotting analysis using the indicated total p53 (DO-1 and pAb421) and phosphospecific-p53 (Ser15p, Ser20p and Ser46p); anti-$p21^{Cip1/Waf1}$ (F-5); anti-Noxa (114C307) antibodies.

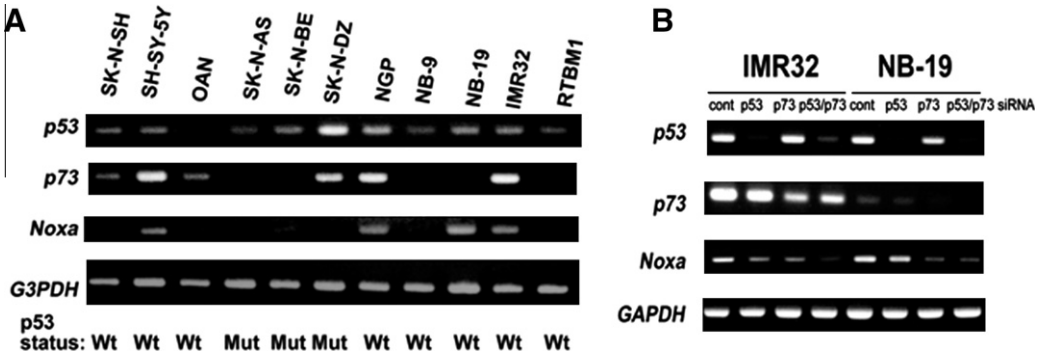

Fig. 5 – Noxa transcription in steady state is regulated by both p53 and p73. (A) Semi-quantitative RT-PCR analysis of p53, p73 and Noxa in NB cells. Total RNA extraction, cDNA synthesis and semi-quantitative RT-PCR were performed. For p53 status, Wt: wild-type; Mut: mutant. (B) Effect of p53 and/or p73 on Noxa transcription. NB cells were transfected with p53 and/or p73 siRNAs. Silencer® Negative control #1 siRNA was used as a control siRNA. Expression of *p53*, *p73* and *Noxa* mRNA were determined by semi-quantitative RT-PCR experiments. The results are representative of at least three independent experiments.

knockdown indicated a synergistic effect on the reduction. In NB-19 cells, p73 seems to have an important role in the regulation of the steady-state *Noxa* mRNA level. Together, these results indicate that Noxa transcription in the steady state was controlled by both p53 and p73 in a complex manner in NB cells.

3.6. *In vivo binding of p53 and p73 to Noxa promoter was suppressed and Noxa promoter activation was inhibited by HDM2*

We studied the *Noxa* mRNA expression after Doxo treatment in HDM2-expressing NB cells, SK1, and found that both

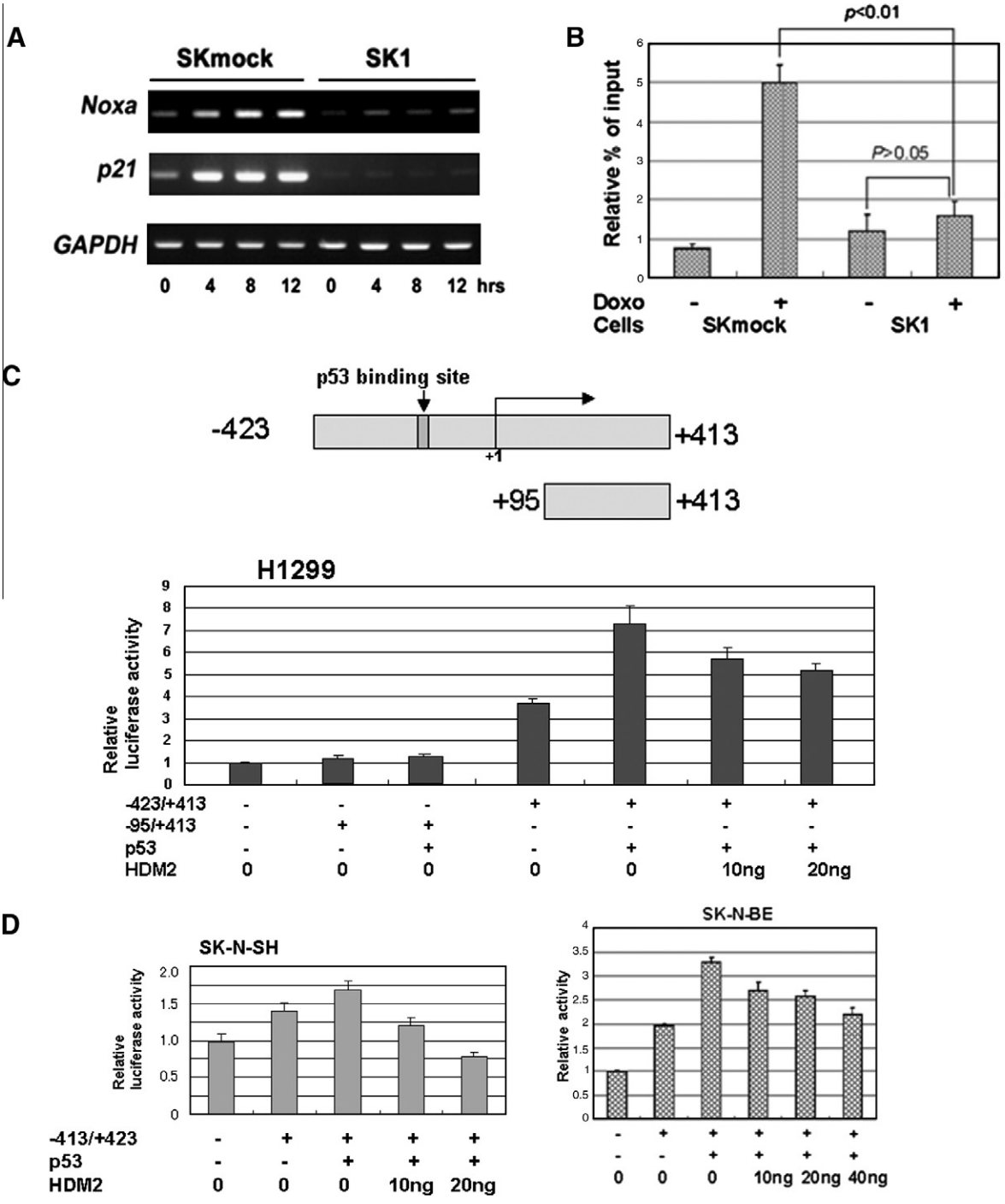

Fig. 6 – HDM2 inhibits p53 *in vivo* binding to Noxa promoter and represses Noxa transcription. (A) *Noxa/p21*$^{cip1/waf1}$ mRNA expression in HDM2-overexpressing NB cells. Cells were treated with 0.3 μg/ml Doxo and collected at the indicated time points. Total RNA extraction, cDNA synthesis and semi-quantitative RT-PCR were performed. (B) Quantitative ChIP assay was performed 16 h after Doxo treatment. Cross-linked chromatin prepared from the indicated cells was precipitated with normal mouse IgG or monoclonal anti-p53 antibody (DO-1). (C and D) Luciferase assay analysis of Noxa promoter activity in H1299, SK-N-SH and SK-N-BE cells. The −423/+413 and +95/413 Noxa fragment information are described in Section 2. pCDNA3-p53 and/or pCMV-HDM2 were transfected with the indicated amounts, and luciferase activity were studied.

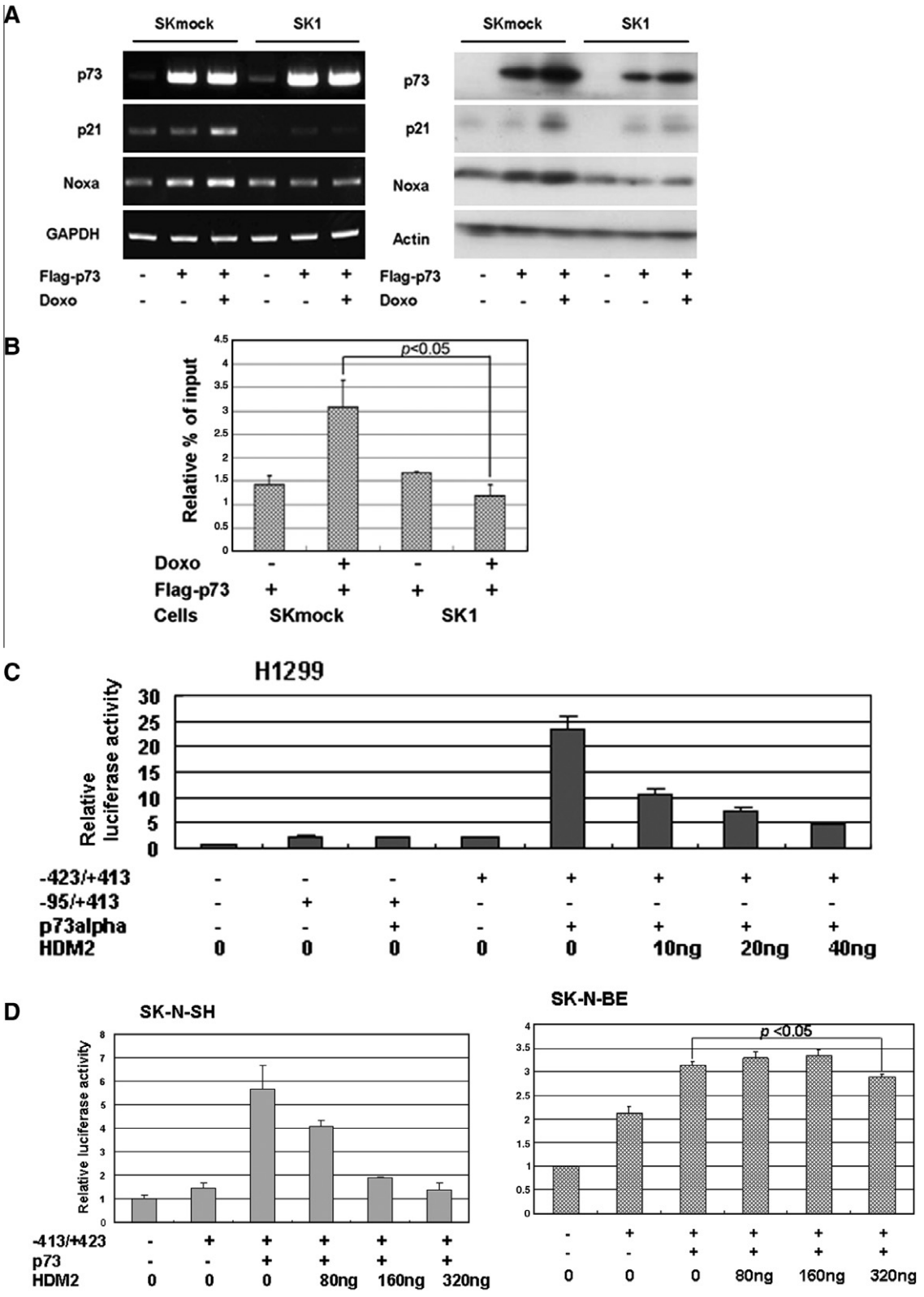

Fig. 7 – HDM2 inhibits p73 binding to Noxa promoter and represses Noxa transcription. (A) *Noxa/p21*$^{cip1/waf1}$ mRNA expression in HDM2/p73 (FLAG-p73α)-overexpressing NB cells. Total RNA was extracted 3 h after 0.3 μg/ml Doxo treatment, and then subjected to cDNA synthesis and semi-quantitative RT-PCR. (B) quantitative ChIP assay of p73 binding to Noxa promoter in NB cells. pCDNA3-FLAGp73 α was transfected into the indicated cells and ChIP assay was performed 12 h after Doxo treatment. Normal mouse IgG or monoclonal anti-FLAG was used to precipitate cross-linked chromatin. (C and D) Luciferase assay analysis of Noxa promoter activity in H1299, SK-N-SH and SK-N-BE cells. Luciferase activity was studied after pCDNA3-FLAGp73 α or/and pCMV-HDM2 transfection with the indicated amounts.

$p21^{Cip1/Waf1}$ and Noxa transcription were significantly impaired (Figs. 4E and 6A). p73-related induction of $p21^{Cip1/Waf1}$ and Noxa was also decreased by HDM2 co-expression (Fig. 7A). Chromatin immunoprecipitation experiments of the Noxa promoter region demonstrated that HDM2 inhibited p53 and p73 *in vivo* binding to the Noxa promoter (Figs. 6B and 7B). The HDM2-induced repression of Noxa promoter activity induced by p53 and p73 was confirmed by luciferase promoter activity measurements not only in lung cancer H1299 cells but also in SK-N-SH and SK-N-BE NB cells (Figs. 6C and 7C).

4. Discussion

4.1. *Transcriptional regulation of Noxa*

Previously, we reported the significance of Noxa in Doxo- and Etopside-related apoptotic cell death in p53 wild-type NB cells.[8] In the present report, we studied the *Noxa* mRNA expression level in steady state and DNA damage-induced NB cells, indicating that the role of p53 seems to be critical for *Noxa* mRNA expression in steady-state NB cells. Furthermore, since Noxa transcription in the steady state was effectively downregulated by p53- or p73-KD in NB cells, it indicated the possible regulation of Noxa transcription in steady-state NB cells not only by p53 but also by p73. Direct binding of p73 to the Noxa promoter and the induction of Noxa promoter activity by p73 confirmed the above-described findings.

Meanwhile, upregulation of *Noxa* mRNA expression by DNA damage was significantly inhibited in functionally p53-inactivated NB cells,[8] suggesting that functional p53 inactivation in p53 wild-type NB cells has a role in Noxa expression in DNA damage-induced stress. Given that p73 was expressed and Doxo treatment could not induce Noxa transcription in p53-mutated SK-N-DZ cells, the mutant p53 may have a considerable suppressive function in Doxo-induced neuroblastoma apoptotic cell death.

Lau et al. reported that HDM2 binds to and inhibits p73, and the dissociation of p73 and HDM2 leads to increased p73 transcriptional activity with upregulation of p73 target genes and enhanced apoptosis.[30] In our experiments, we also observed HDM2-related suppression of p73 transcriptional activity in NB cells by luciferase assay and RT-PCR of p73-downstream targets. These results suggest that HDM2 inhibition of p73 transcriptional activity may have a role in p73-expressing NB cells.

4.2. *Inactivation of p53/Noxa pathway by HDM2 in NB cells*

In the present paper, we studied the molecular mechanism of the functional inactivation of p53 in NB and found that HDM2 was highly expressed and associated with p53 in resistant NB cell lines, although it could not degrade p53 efficiently in those cells. HDM2 seemed to have a role in p53 accumulation in NB cells since both endogenous HDM2 in resistant NB cells and over-expressed HDM2 in SK-N-SH resulted in the failure to accelerate p53 degradation; HDM2 siRNA treatment recovered Noxa expression and sensitivity to Doxo treatment in a p53-dependent manner. Ubiquitination of p53, the dependence of p53 ubiquitination on HDM2 and E3 ubiquitin ligase activity of HDM2 in resistant NB cells were comparable to those in sensitive NB cells (a personal communication by Dr. C. Uchida and Dr. M. Kitagawa, Hamamatsu University). Moreover, a proteasome inhibitor MG132 could not induce the accumulation of p53 in both Doxo-resistant NB-19 and IMR32 cells and HDM2 over-expressing NB cells, indicating that p53 degradation may be independent of the proteasome pathway in Doxo-resistant NB cells. These results suggest the existence of inhibitory proteins which may bind to HDM2 or HDM2/p53 complex and affect p53 accumulation and activation. A candidate for these inhibitors is p14ARF. ARF tumour suppressor binds to HDM2 and inhibits p53 degradation; furthermore, it binds not only to HDM2 but also to p53,[25] however, the expression of ARF in NB cells was very low in several cell lines, and immunoprecipitation of HDM2 indicated only a modest amount of ARF association (data not shown). Taken together, these observations indicate the importance of screening the associated proteins of HDM2 and/or p53 in NB cells to understand the molecular mechanism of p53 inactivation and inhibition of its degradation. Currently, we are searching for these proteins by purification of the tagged-p53 binding protein complex in NB cells.

In conclusion, the present study indicates the following findings: (1) HDM2 plays a role in the functional inactivation of p53 and apoptotic cell death in p53 wild-type NB cells and (2) Noxa transcription is regulated by both p53 and p73 in the steady state and p53 plays an important role in Noxa induction and NB cell apoptotic death after doxorubicin treatment. Thus, our present findings suggest that the HDM2/p53/p73/Noxa pathway has a significant role in the drug resistance of p53 wild-type NB cells and could be a promising candidate for molecular target therapy of advanced-stage NB.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.05.026.

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