Identification and Characterization of Two Splicing Variants of Human Noxa

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Abstract. *Noxa is a pro-apoptotic Bcl-2 homology 3 (BH3)-only containing protein. Here we report the identification of two splicing variants of the human Noxa gene, which consists of three exons and two introns. Alternative splicing of exon 2 yields three transcripts. Transcript-1 joins exons 1 and 3 to encode Noxa of 54 amino acids. Transcript-2, consisting of exon-1, partially spliced exon-2, and exon-3, encodes NSV-1 (Noxa splicing variant-1) of 136 amino acids, whereas transcript-3, containing all three exons, encodes NSV-2 of 70 amino acids, which is 100% identical to the first 70 amino acids of NSV-1. All three transcripts, controlled by the same promoter with two p53 consensus-binding sites, were inducible by p53. Although NSV-1 and NSV-2 mRNA were readily detectable by RT-PCR in multiple human cancer cell lines, no endogenous variant proteins were detected under physiological or stressed conditions. Even under forced expression, both variants were barely detectable without proteasome inhibitor, MG132. The protein half-life of the two variants is approximately 40 to 60 mins with MG132, suggesting they are rapidly degraded via proteasome-dependent and independent pathways. Unlike Noxa, BH3-less variants failed to potentiate apoptosis induced by etoposide. Thus, Noxa variants are unlikely to play a role in apoptosis regulation.*

Apoptosis is a genetically programmed process of cell death required for maintaining homeostasis under physiological conditions and for responding to various internal and external stimuli (1-3). Cells committed to apoptosis are characterized by membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation and DNA fragmentation (4). Two major apoptosis signaling pathways have been defined in mammalian cells. The extrinsic death-receptor pathway is triggered by members of the death receptor superfamily, followed by recruitment of Fas-associated death domain (FADD) protein and activation of caspase-8, then caspase-3. The intrinsic mitochondrial pathway is activated in response to extracellular cues and internal insults, such as DNA damage, leading to cytochrome C release to the cytoplasm, followed by the formation of apoptosomes and activation of caspase-9 and then caspase-3 (5). Both apoptotic pathways converge at the activation of caspase-3, which then cleaves its substrates, leading to apoptotic cell death (6, 7).

Among many molecular players that regulate apoptosis, Bcl-2 family members play a critical and essential role, particularly in the intrinsic mitochondrial pathway (8, 9). Bcl-2 family members consist of both anti-apoptotic and pro-apoptotic proteins.

Anti-apoptotic members, including Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1, contain four Bcl-2 homology (BH) domains. The pro-apoptotic members can be subdivided into two groups: the Bax subfamily (Bax, Bak and Bok), containing BH1, BH2, and BH3 domains, and BH3-only proteins, such as Bid, Bad, Bim and Noxa (10). Anti-apoptotic members of the Bcl-2 family are often anchored to membranes of mitochondria and endoplasmic reticulum to block the release of mitochondrial apoptogenic factors, such as cytochrome C and Smac, whereas many pro-apoptotic members are normally found in the cytosol and/or are loosely attached to membranes. In response to environmental stimuli, Bax inserts into the mitochondrial outer membrane as homo-oligomers and induces release of cytochrome C and Smac to initiate apoptosis signaling pathway, which is inhibited by Bcl-2 (8, 11).

Noxa is a BH3-only pro-apoptotic protein that specifically inhibits the anti-apoptotic Bcl-2 family member, Mcl-1 (12, 13) through promoting its degradation (14, 15). *Noxa* is a p53 target gene. In response to apoptotic stimuli, Noxa is induced by p53 and mediates p53-induced apoptosis (16). Noxa is also found to be induced by hypoxia-inducible factor (HIF-1)

Abbreviations: BH3, Bcl-2 homology 3; NSV, Noxa Splicing Variants; PMA-IP1, phorbol-12-myristate-13-acetate-induced protein 1.

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and mediates HIF-1 induced hypoxic cell death (17). The importance of Noxa in apoptosis promotion is further supported by Noxa knockout studies, which showed that Noxa-null fibroblasts are more resistant to DNA damage-induced apoptosis (18) and that Noxa is responsible for UV-induced apoptosis in skin keratinocytes and fibroblasts (19). Furthermore, Noxa was recently found to be induced by proteasome inhibitor, PS341 and mediates PS341-induced apoptosis in a number of types human cancer (20-23).

Human Noxa, also designated PMA-IP1 in the database, consists of 54 amino acids with one BH3 domain (NM_021127), whereas mouse Noxa consists of 103 amino acids with two BH3-only domains (16). No splicing variants of human Noxa were previously described. Here we found that the human *Noxa* gene encodes three splicing variants: *Noxa, Noxa splicing variant-1 (NSV-1, or PMA-IP1)* and *NSV-2*. Both variants lack the pro-apoptotic BH-3 domains. In this report, we aim to characterize these two splicing variants with regards to their inducibility by p53, their protein half-lives, and their biological function in response to DNA damaging agent, in comparison with Noxa.

Materials and Methods

Cell culture. Human cancer cell lines, including H460, H1299, 293, U2-OS, MCF-7, SY5Y, DLD-1, A549, T47D and HeLa (from American Type Culture Collection, ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum in an incubator at 37˚C.

Total RNA isolation and RT-PCR analysis. Total RNA was isolated from H460 or H1299 cells using a Trizol kit (Invitrogen, Carlsbad, CA, USA), and subjected to RT-PCR analysis (Titan OneTube RT-PCR, Roche, Basel, Switzerland). The primer sequences for RT-PCR amplification of *Noxa* were Noxa-F1 (with FLAG-tag coding sequence at the 5'-end): 5'-ccCTCGAGGCCACCATGGACT ACAAGGACGACGATG ACAAGCCTGGGAAGAAGGCGCGC-3' and NSV1-R1: 5'-cggGGTACCTCAATTA CAATGCAGT CTTTC to generate a PCR fragment of 294 bp. The primers used to simultaneously amplify both *NSV-1* and *NSV-2* were Ex02F: 5'-GACCAAGCCG GATTTGCGAT-3' and NSV1-R1, generating the fragments with a size of 460 bp (NSV-2) and 324 bp (NSV-1), respectively. The identity of these fragments was confirmed by DNA sequencing. For loading control, the house keeping gene, *GAPDH*, was included in RT-PCR analysis; the primers used were GAPDH-01, 5'-GTTGCCATCAATGACCC CTT-3' and GAPDH-02, 5'-AGAGGCAGGGATGATGTTCT-3' to generate a PCR fragment of 540 bp.

Western blotting analysis. Cells were harvested after compound treatment and lysed using Triton lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton, 5 mM EGTA and 5 mM EDTA and proteinase inhibitors). The supernatants were collected by centrifugation and subjected to SDS-PAGE, followed by transfer onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). The membranes were blocked with 5% Blotto and probed with antibodies against FLAG and β-actin (Sigma, St. Louis, MO, USA) and NSV, followed by visualization by ECL (Pierce, Rockford, IL, USA) detection kit. Poly-clonal rabbit NSV antibody was generated against a peptide (CSSSPLALPRGHEEQ) (Amino Acids 54-67 of NSV-1 or -2) with affinity purification by YenZym Antibodies, LLC (Burlingame, CA, USA).

cDNA cloning and transient transfection. Noxa and its two splicing variants were cloned into pcDNA3 vector by PCR amplification and subcloning. The primers used for Noxa cloning were Noxa-F1 and Noxa-R1: 5'-cggGGTACCTCAGGTTCCTGAGCAG AAGA-3. A two-step PCR reaction was conducted to generate the entire open reading frame of NSV-1 and NSV-2. The template-1 was generated by PCR amplification using primer set of Noxa-F1 and Ex02R: 5'-TTGCACTTGTTCCTCGTGGCC-3' and the template-2 with primer set of Ex02F and NSV1-R1. Two templates were combined in a PCR reaction to generate NSV-1 coding cDNA by the primer set of Noxa-F1 and NSV1-R1, and NSV-2 coding cDNA by the primer set Noxa-F1 and NSV2-R1: 5'-cggGGTACCTACACTTGCACTTGT TCC TC-3', respectively. The PCR fragments were digested with KpnI and XhoI (New England Biolabs, Ipswich, MA, USA) and directly cloned into pcDNA3 (–), followed by confirmation by DNA sequencing. The DNA was transiently transfected into 293 cells, using TR01 reagent (America Pharma Source, Rockville, MD, USA).

FACS analysis. The 293 cells were transiently transfected with plasmids encoding Noxa, NSV-1, and NSV-2, along with pcDNA3 empty vector negative control. Twenty-four hours post-transfection, cells were left untreated, or treated with MG132 (5 μM) alone or in combination with etoposide (50 μM) for 24 h. Both detached and attached cells were then harvested with trypsin (Invitrogen) and fixed in 70% EtOH at –20˚C for at least 4 h, suspended in 1× propidium iodide solution with 400 μg/mL RNase (Roche, Basel, Switzerland), and analyzed in the Flow Cytometry Lab facility at the University of Michigan (24). The fraction of cells in the sub-G_1 peak was considered as the percentage of apoptosis.

Results

Identification of Noxa splicing variants. We have previously performed a genome-wide chip expression profiling and identified few thousand genes responsive to p53-induced growth arrest and apoptosis using a p53 temperature sensitive human lung carcinoma cell model (25). One gene, designated as *phorbol-12-myristate-13-acetate-induced protein 1* (*PMA-IP1*, accession No. AI857639) was identified as a responder to both p53-induced growth arrest and apoptosis (25). BLASTn search using this IMAGE cDNA clone as the bait identified 1.28 kb cDNA encoding human PMA-IP1 (Accession No. BC032663). *PMA-IP1* was initially identified as a PMA-responsive immediate early gene from acute T cell leukemia (26), which was also designated mistakenly as *Noxa* in the database. Direct sequence comparison between *PMA-IP1* (BC032663) and *Noxa* (Accession No. NM_021127) revealed that in the coding region, *PMA-IP-1* contains a 151-bp fragment immediately downstream of exon 1, which is absent in

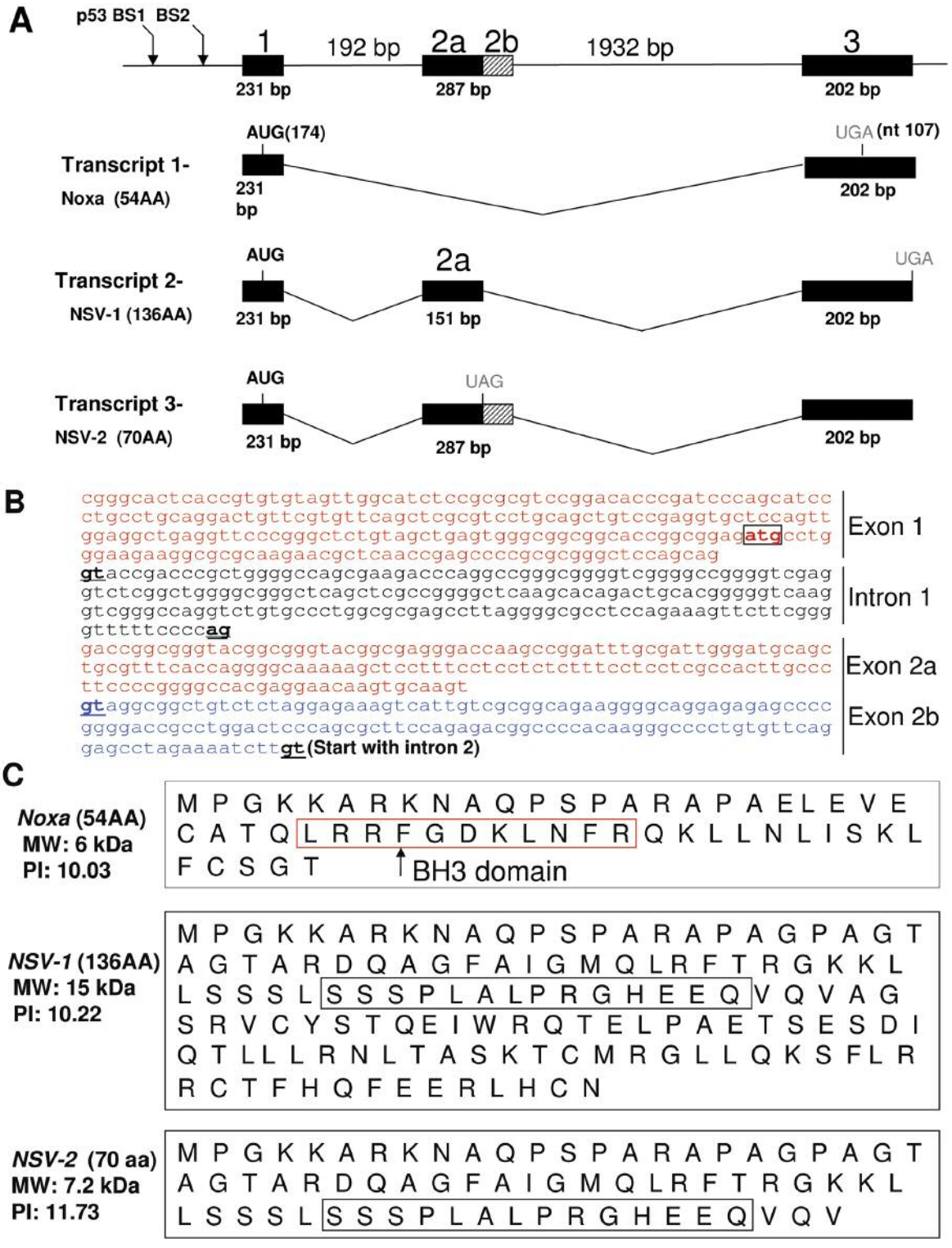

Figure 1. *Identification of human Noxa splicing variants. A, The genomic structure of the human Noxa gene with three transcripts derived from alternative splicing. The gene contains three exons and two introns with the size indicated. The translation initiation site and termination site were labeled for each transcript, along with three different types of splicing. Drawing is not to scale. Two p53-binding sites (BS) are indicated in the promoter region. B, DNA sequence of exon-1, intron-1 and exon-2a/2b with exon-intron boundary sequence underlined. C, Predicted amino acid sequences, calculated molecular weights, and pI values of Noxa and its two splicing variants. The BH3 domain as well as the peptide sequence used to generate anti-NSV antibody are highlighted in the boxes.*

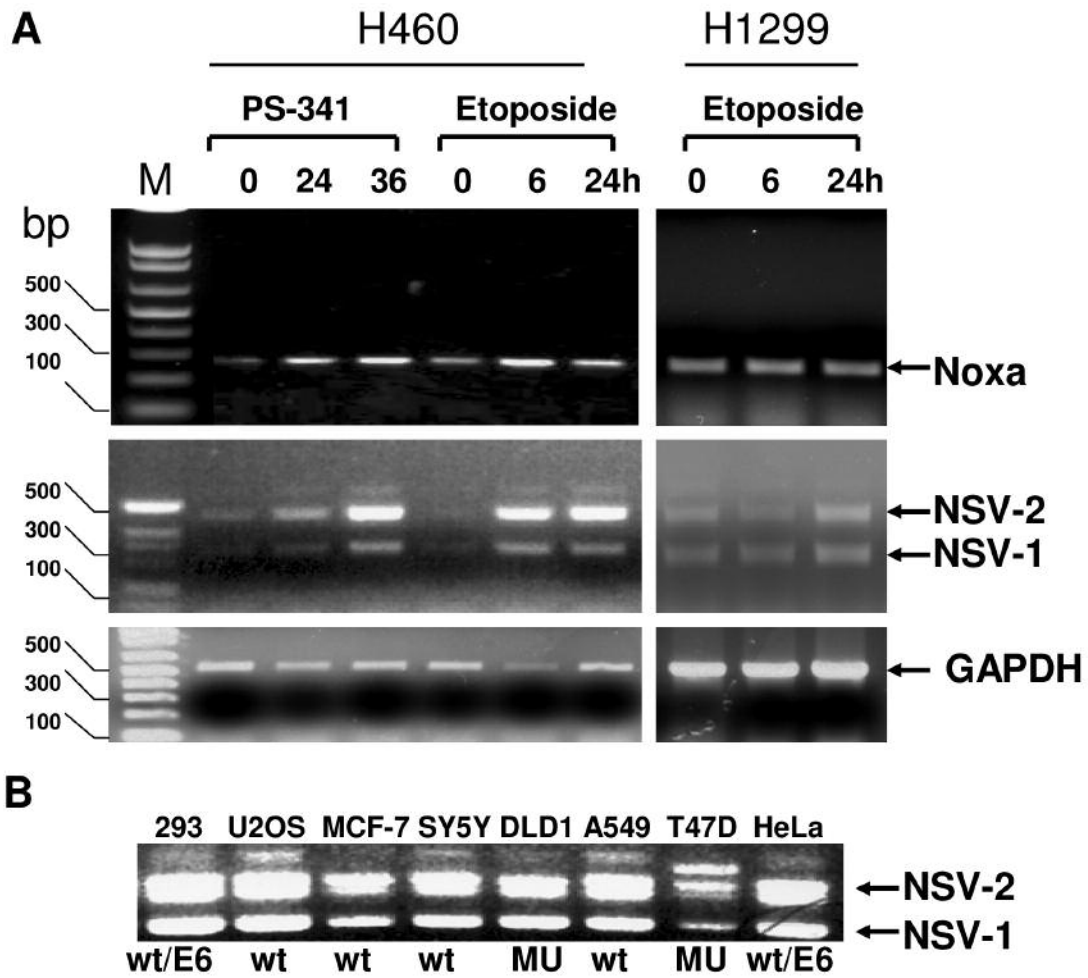

Figure 2. *Detection by RT-PCR of all three alternative spliced transcripts of human Noxa. A, Induction of Noxa transcripts by PS341 and etoposide: Total RNAs were isolated from H460 and H1299 cells after treatment with PS341 (50 nM) or etoposide (25 μM) for periods of time indicated and subjected to semi-quantitative RT-PCR analysis, followed by agarose gel electrophoresis, as described in Materials and Methods. PCR fragments of each variant are indicated with their expected size. B, Detection of NSV-1 and NSV-2 mRNA in variety of human cancer cell lines: Subconfluent cells were harvested and subjected to RNA isolation and semi-quantitative RT-PCR analysis. The fragments with expected size were visualized after agarose gel electrophoresis and ethidium bromide staining. The p53 status of each line was indicated below: wt, wild type p53; MU, mutant p53; wt/E6, wild type p53 in geneome, but lines were contaminated with papillomaviral E6 oncoprotein that degrades p53.*

Noxa, suggesting *PMA-IP1* is a splicing variant of *Noxa* with shifted open reading frame. Using this 151-bp fragment as the bait, the EST Database search identified yet another related IMAGE clones (Accession No. BG393278) with additional unique 136-bp fragment immediately downstream the 151-bp bait sequence, suggesting the possibility of yet another splicing variant. The coding sequence of all three clones was subjected to BLASTn search individually against human genome. Analysis of the search results revealed that the *Noxa* gene is located in human chromosome 18q21.32 (Accession No. AC090377) and contains three exons and two introns (Figure 1A, top bar structure, with size of each exon and intron indicated). *Noxa* is designated as transcript 1 with exon 2 completely spliced out. *PMA-IP1* is designated as transcript 2 (*Noxa splicing variant 1, NSV-1*) with exon 2b spliced out, whereas the transcript 3, designated as *NSV-2*, contains all three exons (Figure 1A). The sequence of exon-1-intron-1-exon-2 of human *Noxa* gene is listed in Figure 1B. Conventional intron starting (gt) or ending (ag) sequences are conserved and underlined. The open reading frame of *Noxa* consists of 165-bp and encodes a peptide of 54 amino acids with one BH3 domain (boxed in Figure 1C). *NSV-1* has an open reading frame of 411-bp, encoding a peptide of 136 amino acids, whereas *NSV-2* has an open reading frame of 213-bp to encode a peptide of 70 amino acids, which is exactly the same as the N-terminal portion of NSV-1 (Figure 1C). All three variants contain the same first 19

amino acids encoded by exon-1. Due to frame shift, NSV-1 and NSV-2 do not contain pro-apoptotic BH3 domain. Analysis of genomic sequence of mouse *Noxa* gene (AB041230) revealed that it contains 3 exons and 2 introns as well. The open-reading frame of mouse *Noxa* consists of all three exons to encode a protein of 103 amino acids with two BH3 domains (16).

Induction of Noxa and two NSV mRNAs by proteasome inhibitor, PS341, and DNA damage agent, etoposide. It is well-established that Noxa is a transcriptional target of p53 and is also subjected to PS341 up-regulation at both mRNA and protein levels (16, 20). Indeed, we identified two p53-binding sites at the promoter of human *Noxa* gene, upstream of exon-1 at –194 to –175 (5'-GAGCgTGTCCGGGC AgGTCg-3') and –456 to –437 (5'-GGGCTTGTTTAccCAA GTCT-3'), respectively (small case for mismatch with consensus p53-binding site). We next asked whether like *Noxa*, *NSV-1* and *NSV-2* transcripts were also subjected to up-regulation by PS-341 and p53-inducing DNA damaging agent, etoposide. As shown in Figure 2A, top panel, RT-PCR analysis revealed that in wild type p53-containing H460 cells, both PS-341 and etoposide caused a significant induction of *Noxa*. Similarly, both *NSV-1* and *NSV-2* was also dramatically induced by two agents (Figure 2A, middle panel). Induction of all three *Noxa* transcripts appears to be p53 dependent since no induction can be found in p53-null H1299 cells by etoposide (Figure 2A). The housekeeping gene, *GAPDH* was included in the assay as a loading control (Figure 2A, bottom panel). Thus, p53 appears to bind to its consensus sequences at the promoter of human *Noxa* and transactivates the expression of all three transcripts. To determine whether *NSV-1* and *NSV-2* are also expressed in additional human cancer cell lines, we used RT-PCR analysis and showed that expression of both variants were readily detectable with *NSV-2* level relatively higher in multiple human cancer cell lines, regardless of p53 status (Figure 2B).

Expression of Noxa splicing variant proteins. In an attempt to measure the endogenous levels of NSV-1 and NSV-2 proteins and to determine whether they are subjected to PS-341 or p53 induction, we generated a polyclonal antibody against the C-terminal 14-amino acid peptide of NSV-2 (Figure 1C, boxed sequence). The antibody (α-NSV) is, therefore, specific for two Noxa variants, but not for Noxa itself. This specific affinity-purified antibody, however, failed to detect two variants in multiple human cancer cell lines listed in Figure 2B even after treatment with PS-341 or etoposide (data not shown). It appears that two variants are expressed at undetectable levels even after exposure to their inducers. To further determine their potential expression in mammalian cells, both variants were cloned, with a FLAG-tag placed at the N-terminus, into mammalian expression

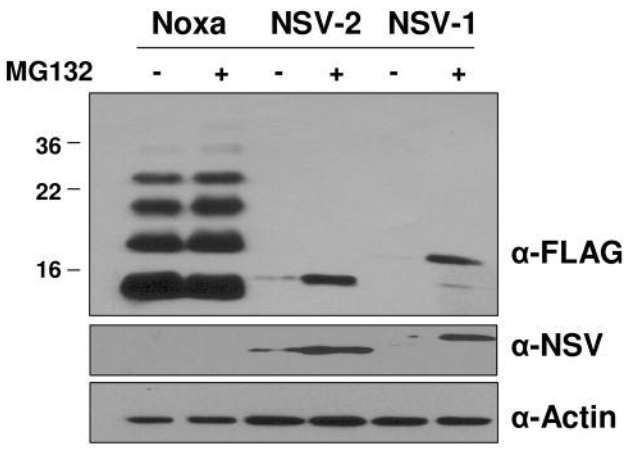

Figure 3. *Protein expression of Noxa and its splicing variants after transient transfection. The 293 cells were transiently transfected with plasmid expressing FLAG-tagged Noxa, NSV-1 and NSV-2, respectively. Cells were harvested 48 h post-transfection. In some dishes, proteasome inhibitor, MG132 (10 μM) was added 8 hours prior to harvest. Cell lysates were prepared and subjected to Western blotting analysis using antibodies against FLAG-tag or NSV. β-Actin was used as an internal loading control.*

vector, pcDNA3, along with FLAG-tagged Noxa as positive control. All three constructs were transiently transfected into 293 cells. Cells were harvested 48 h post transfection with or without treatment of proteasome inhibitor, MG132 in last 8 h, followed by western blotting analysis using anti-FLAG antibody. As shown in Figure 3, top panel, Noxa expressed at the very high levels mainly in a monomeric form as well as in di-, tri- and tetra-meric forms. Inclusion of MG132 had no effect. In contrast, both NSV-1 and NSV-2 expressed at barely detectable levels, which were significantly increased by MG132 treatment, suggesting that both Noxa variants underwent rapid degradation after expression in a proteasome dependent manner. The similar results were obtained when NSV specific antibody, α-NSV was used (Figure 3, middle panel). The extremely low levels of expression prevented us from generating stable clones for both Noxa variants (data not shown).

NSV-1 and NSV-2 have a very short protein half-life even in the presence of MG132. We next determined the relative half-life of two variants, as compared to Noxa in 293 cells after transient transfection. Cells at 36 h post-transfection were treated with cycloheximide (CHX) to block new protein synthesis and harvested at 2, 4, 6, 8 and 24 h thereafter for Western Blotting analysis. As shown in Figure 4A, both variants were barely detectable even before CHX addition and completely disappeared at 2 h post CHX treatment. In contrast, Noxa was expressed at very high levels in forms of monomer and oligomers with a protein

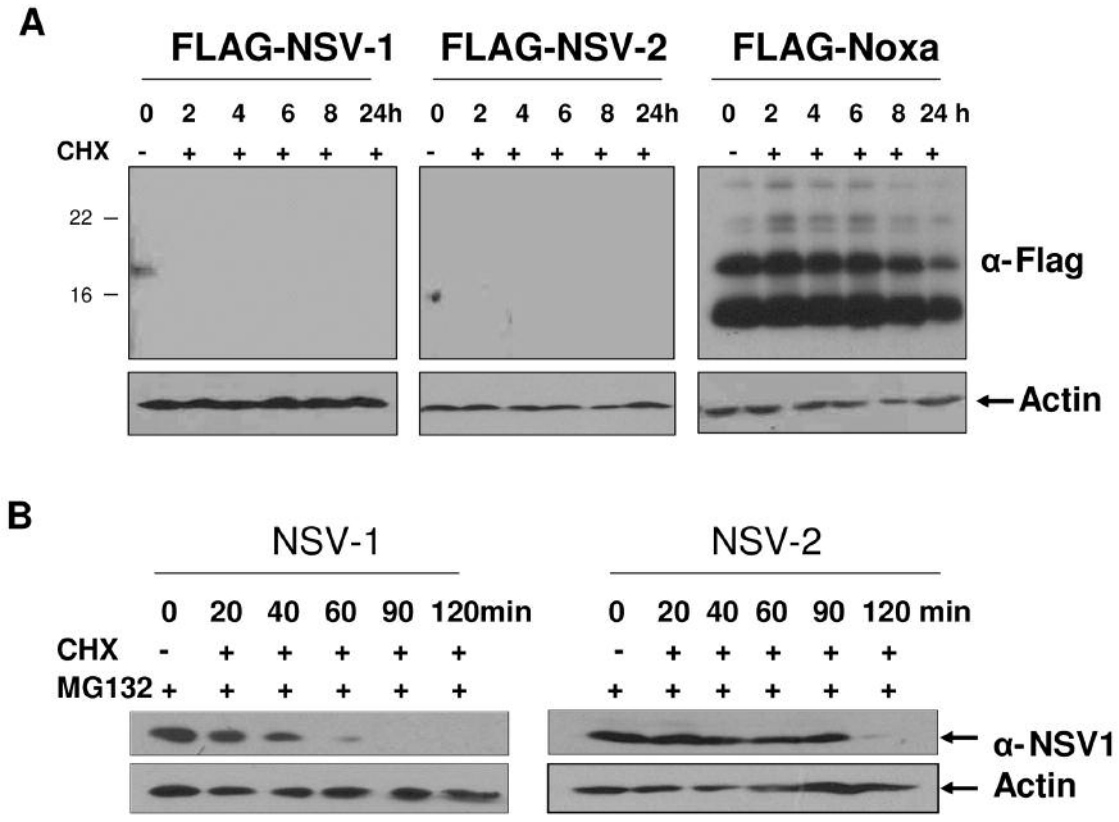

Figure 4. *Noxa variants have short protein half-life: The 293 cells were transiently transfected with plasmids expressing FLAG-tagged Noxa and its two variants, respectively. Twenty-four hours post-transfection, a protein synthesis inhibitor, cycloheximide (CHX, 50 μg/mL), was added alone (A) or in combination with MG132 (10 μM, added 4 h prior to CHX) (B). Cells were harvested at indicated time periods thereafter and subjected to Western blotting analysis using antibody against FLAG-tag with β-actin as a loading control. Representative figures from two independent experiments are shown.*

half-life approximately 24 h. Since MG132 treatment can significantly increase the levels of both NSV-1 and NSV-2 (Figure 3), we determined their protein half-life in the presence of proteasome inhibitor. As shown in Figure 4B, NSV-1 had a half-life of about 40 min, whereas NSV-2 of approximately 60 min. Such a short half-life even in the presence of proteasome inhibitor indicated a proteasome independent degradation. Thus, upon translation, Noxa variants are subjected to rapid degradation by both proteasome-dependent and -independent pathways. The extremely short protein half-life of variants may account for their undetectable endogenous as well as inducible levels.

Unlike Noxa, both variants have no apoptosis-promoting activity. We next tested our working hypothesis is that due to the lack of BH3 domain in two splicing variants; they are unlikely to mediate p53-induced apoptosis, although being induced by p53. We transfected two variants into 293 cells individually, along with empty vector as negative control and Noxa as positive control. Cells, after 24 h transfection, were left untreated, treated with MG132, or treated simultaneously

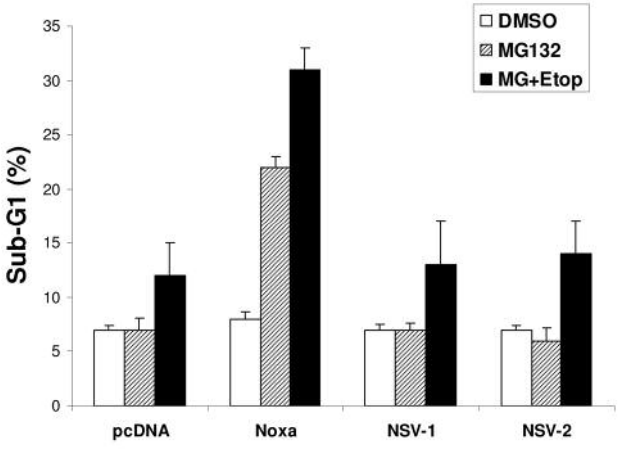

Figure 5. *BH3-less variants failed to potentiate etoposide-induced apoptosis. The 293 cells were seeded in 12-well plates and transfected with 2 μg of plasmids expressing NSV-1, NSV-2 or Noxa individually. Twenty-four hours post-transfection, cells were treated with MG132 (5 μM) alone or in combination with etoposide (50 μM) for an additional 24 h. Both detached and attached cells were then harvested and prepared for FACS analysis, as described in Materials and Methods. Data represent means ± SD from two independent experiments.*

with MG132 (to remain the expression of variants, see Figure 3) and etoposide (to induce apoptosis) for 24 h. Both detached and attached cells were harvested and subjected to FACS analysis for apoptosis induction. As shown in Figure 5, Noxa over-expression alone did not induce measurable apoptosis in 293 cells, but combination of Noxa over-expression and MG132 treatment increased apoptotic population from the basal level of 5% to 23% . Addition of etoposide further increased apoptotic population to 32% . In contrast, expression of both variants (in the presence of MG132) had no effect on apoptosis induction. Combination with etoposide did increase apoptotic population from 5% to 13% , but this is comparable to empty vector control. Thus, unlike Noxa, both variants lose apoptosis-promoting activity in response to DNA damaging agent.

Discussion

Noxa is a BH3- only pro-apoptosis protein that promotes apoptotic cell death in both *in vitro* cell culture models and *in vivo* mouse models (16, 18, 19). The therapeutic application of BH3-mimetic compounds as apoptotic inducing, anti-cancer drugs are under intensive investigation and clinic development (27, 28). Alternative RNA processing of the *Noxa* gene, which will add another level of complexity for Noxa regulation, has not been previously reported.

Here, we report the identification of the two human *Noxa* splicing variants that are expressed at the mRNA levels and subjected to induction by p53-inducing agent, etoposide and proteasome inhibitor, PS341. These two *Noxa* variants are unlikely derived from PCR artifacts, since individual cDNA IMAGE EST clones were found for both variants (BC032663 and BG393278). However, we were unable to detect their expression at the protein levels under physiological or stressed conditions, likely due to their extremely short protein half-life. Detection of the two variants in the presence of MG132, indicates that they are subjected to proteasome-dependent degradation. Furthermore, a complete elimination of the two variants within 1-2 h after CHX treatment in the presence of MG132, strongly suggests that they are also subjected to degradation by proteasome-independent pathway, since without CHX, both variants are detectable after 48 hrs post-transfection with MG132 presented in last 8 hrs (Figure 3). Thus, we concluded that two Noxa variants are subjected to rapid degradation by both proteasome-dependent and proteasome-independent pathways.

The finding that both variants lack pro-apoptotic BH3 domain promoted us to test our working hypothesis that both variants, although induced equally well as Noxa by p53, will have no pro-apoptotic activity. This turned out to be the case. Due to their very low levels of expression, we were unable to test whether the two variants would act in a dominant negative manner to block Noxa activity.

In summary, this is the first report that human *Noxa* does have two splicing variants that are induced by p53 and other stress stimulus at the mRNA levels and that *PMA-IP1* is not *Noxa*, but a *Noxa* splicing variant. Due to their extremely unstable nature, both variants are hardly detectable under normal physiological condition or even after exposure to stressed conditions. Thus, it appears that the two Noxa variants are unlikely to play any significant role in the regulation of apoptosis or other biological processes.

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References

1 Kerr JF, Wyllie AH and Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*: 239-257, 1972.
2 Wyllie AH, Kerr JF and Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol *68*: 251-306, 1980.
3 Thompson CB: Apoptosis in the pathogenesis and treatment of disease. Science *267*: 1456-1462, 1995.
4 Wyllie AH: Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature *284*: 555-556, 1980.
5 Hengartner MO: The biochemistry of apoptosis. Nature *407*: 770-776, 2000.
6 Salvesen GS and Dixit VM: Caspases: intracellular signaling by proteolysis. Cell *91*: 443-446, 1997.
7 Cryns V and Yuan J: Proteases to die for. Genes Dev *12*: 1551-1570, 1998.
8 Danial NN and Korsmeyer SJ: Cell death: critical control points. Cell 116: 205-219, 2004.
9 Reed JC, Jurgensmeier JM and Matsuyama S: Bcl-2 family proteins and mitochondria. Bi ochim Biophys Acta *1366*: 127-137, 1998.
10 Gross A, McDonnell JM and Korsmeyer SJ: BCL-2 family members and the mitochondria in apoptosis. Genes Dev *13*: 1899-1911, 1999.
11 Martinou JC and Green DR: Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol *2*: 63-67, 2001.
12 Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM and Huang DC: Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell *17*: 393-403, 2005.
13 Kim H, Rafiuddin-Shah M, Tu HC, Jeffers JR, Zambetti GP, Hsieh JJ and Cheng EH: Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol *8*: 1348-1358, 2006.
14 van Delft MF, Wei AH, Mason KD Vandenberg CJ, Chen L, Czabotar PE, Willis SN, Scott CL, Day CL, Cory S, Adams JM, Roberts AW and Huang DC: The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis *via* Bak/Bax if Mcl-1 is neutralized. Cancer Cell *10*: 389-399, 2006.

15 Czabotar PE, Lee EF, van Delft MF, Day CL, Smith BJ, Huang DC, Fairlie WD, Hinds MG and Colman PM: Structural insights into the degradation of Mcl-1 induced by BH3 domains. Proc Natl Acad Sci USA *104*: 6217-6222, 2007.

16 Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T and Tanaka N: Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*: 1053-1058, 2000.

17 Kim JY, Ahn HJ, Ryu JH, Suk K and Park JH: BH3-only protein Noxa is a mediator of hypoxic cell death induced by hypoxia-inducible factor 1alpha. J Exp Med *199*: 113-124, 2004.

18 Villunger A, Michalak EM, Coultas L, Müllauer F, Böck G, Ausserlechner MJ, Adams JM and Strasser A: p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science *302*: 1036-1038, 2003.

19 Naik E, Michalak EM, Villunger A, Adams JM and Strasser A: Ultraviolet radiation triggers apoptosis of fibroblasts and skin keratinocytes mainly *via* the BH3-only protein Noxa. J Cell Biol *176*: 415-424, 2007.

20 Fernandez Y, Verhaegen M, Miller TP, Rush JL, Steiner P, Opipari AW Jr, Lowe SW and Soengas MS: Differential regulation of noxa in normal melanocytes and melanoma cells by proteasome inhibition: therapeutic implications. Cancer Res *65*: 6294-6304, 2005.

21 Perez-Galan P, Roue G, Villamor N, Montserrat E, Campo E and Colomer D: The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. Blood *107*: 257-264, 2006.

22 Fribley AM, Evenchik B, Zeng Q, Park BK, Guan JY, Zhang H, Hale TJ, Soengas MS, Kaufman RJ and Wang CY: Proteasome inhibitor PS-341 induces apoptosis in cisplatin-resistant squamous cell carcinoma cells by induction of Noxa. J Biol Chem *281*: 31440-31447, 2006.

23 Voortman J, Checinska A, Giaccone G, Rodriguez JA and Kruyt FA: Bortezomib, but not cisplatin, induces mitochondria-dependent apoptosis accompanied by up-regulation of noxa in the non-small cell lung cancer cell line NCI-H460. Mol Cancer Ther *6*: 1046-1053, 2007.

24 Bockbrader KM, Tan M and Sun Y: A small molecule Smac-mimic compound induces apoptosis and sensitizes TRAIL- and etoposide-induced apoptosis in breast cancer cells. Oncogene *24*: 7381-7388, 2005.

25 Robinson M, Jiang P, Cui J, Li J, Wang Y, Swaroop M, Madore S, Lawrence TS and Sun Y: Global Genechip profiling to identify genes responsive to p53-induced growth arrest and apoptosis in human lung carcinomas. Cancer Biol Therapy *2*: 406-415, 2003.

26 Hijikata M, Kato N, Sato T, Kagami Y and Shimotohno K: Molecular cloning and characterization of a cDNA for a novel phorbol-12-myristate-13-acetate-responsive gene that is highly expressed in an adult T-cell leukemia cell line. J Virol *64*: 4632-4639, 1990.

27 Adams JM and Cory S: The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene *26*: 1324-1337, 2007.

28 Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW and Rosenberg SH: An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature *435*: 677-681, 2005.

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