

Nijmegen Breakage Syndrome Protein (NBN) Causes Resistance to Methylating Anticancer Drugs Such as Temozolomide

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ABSTRACT

Methylating agents are first-line therapeutics for gliomas and malignant melanomas. They attack DNA at various sites, and both O⁶-methylguanine and N-methylated base adducts contribute to the killing response. The mechanism of cellular defense against these agents primarily involves O⁶-methylguanine-DNA methyltransferase (MGMT) and base excision repair (BER). Here, we determined whether a key protein involved in DNA double-strand break (DSB) recognition and signaling, nibrin (NBN alias NBS-1), plays a role in the cellular defense against methylating agents. Comparing *NBN* mutated fibroblasts and lymphoblastoid cells from patients suffering from Nijmegen breakage syndrome, we show that *NBN* mutants are clearly more sensitive to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and temozolomide than the corresponding wild-type cells. Hypersensitivity was due to the induction of both apoptosis and necrosis. The mismatch repair proteins MSH2, MSH6, MLH1,

and PMS2 were expressed at a similar level in the cell lines and BER was not affected by *NBN* mutation. Because MGMT expression abrogated the hypersensitivity of *NBN* mutated cells, we conclude that O⁶-methylguanine-derived lesions are responsible for triggering the response. Down-regulation of NBN in melanoma cells by small interfering RNA rendered them more sensitive to temozolomide, suggesting that NBN is a novel modulator of temozolomide sensitivity. Because NBN is part of the MRN complex, which recognizes DSBs, the data strongly indicate that MRN is critically involved in DSB processing after O⁶-methylguanine induction. The data provide first evidence that NBN is involved in the cellular defense against O⁶-methylguanine-inducing agents such as temozolomide and identify NBN as a critical target of methylating anticancer drug resistance.

Introduction

Nibrin, the product of the *NBN* gene (originally designated *NBS-1*), is a protein with an essential function in DNA double-strand break (DSB) repair by both homologous recombination (HR) (Tauchi et al., 2002) and nonhomologous end joining (NHEJ) (Dinkelmann et al., 2009). It is defective in the autosomal recessive chromosomal instability disorder Nijmegen Breakage Syndrome (NBS) (Varon et al., 1998). Nibrin forms a complex with MRE11 and RAD50, known as the MRN complex, and is required for nuclear localization of the complex and its translocation to DSBs (Desai-Mehta et al.,

2001). MRN stimulates phosphorylation of H2AX and ATM. Nibrin itself is also phosphorylated by the ATM kinase (Gatei et al., 2000); therefore, NBN acts both upstream and downstream of ATM. NBN is also involved in ATR function (Stiff et al., 2005), regulation of cell cycle checkpoint control (Lee et al., 2003), and apoptosis signaling (Sagan et al., 2007).

NBN is frequently mutated in glioblastoma multiforme (WHO grade IV) (Watanabe et al., 2009) and medulloblastomas (Huang et al., 2008). It is overexpressed in uveal melanomas, correlating with metastatic death, and was therefore suggested as a prognostic marker for melanomas (Ehlers and Harbour, 2005). This, and the key role in DSB recognition and repair, makes NBN a clinically highly interesting protein. Because glioblastomas and malignant melanomas are routinely treated with methylating agents such as temozolomide (TMZ) (Stupp et al., 2005) and dacarbazine

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ABBREVIATIONS: DSB, double-strand break; HR, homologous recombination; NHEJ, nonhomologous end joining; NBN, nibrin; NBS, Nijmegen Breakage Syndrome; TMZ, temozolomide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; O⁶MeG, O⁶-methylguanine; O⁶BG, O⁶-benzylguanine; MMR, mismatch repair; MGMT, O⁶-methylguanine-DNA methyltransferase; BER, base excision repair; wt, wild type; mt, mutant; siRNA, small interfering RNA; O⁶BG, O⁶-benzylguanine; PI, propidium iodide; PARP, poly (ADP-ribose) polymerase; WST, water-soluble tetrazolium.

(Middleton et al., 2000), it is important to elucidate whether NBN plays a role in tumor cell killing induced by this type of chemotherapeutics.

Methylating agents, such as the model S_N1 mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and the anticancer drug temozolomide, methylate DNA at 13 positions (Beranek, 1990; Kaina et al., 2007). Although accounting for less than 8% of total base modifications, methylation of guanine at the O^6 -position is most critical because it is mutagenic and cytotoxic. The toxicity of O^6 -methylguanine (O^6 MeG) is dependent on DNA mismatch repair (MMR) (Karran and Bignami, 1994) and DSB formation (Ochs and Kaina, 2000; Roos et al., 2009a), which occurs in the second S-phase after its induction (Quiros et al., 2010). The model of toxicity states that in the first DNA replication cycle O^6 MeG mispairs with thymine. This activates MutS α -dependent MMR (Mojas et al., 2007), which leads to removal and reinsertion of the mispaired base. This futile MMR cycle will finally impair DNA replication and may lead to collapse of replication forks and the formation of DSBs, which have been demonstrated by both the neutral comet assay (Ochs and Kaina, 2000) and H2AX phosphorylation (Roos et al., 2009a). Toxicity is mainly executed by apoptosis (Kaina et al., 1997) very likely triggered by DSBs. MMR itself may also trigger genotoxic signaling (Yoshioka et al., 2006). Therefore, the possibility remains that in some cell types, the activated MMR complex triggers cell death directly.

O^6 -Methylguanine-DNA methyltransferase (MGMT) protects against O^6 MeG triggered toxicity by removal of the methyl residue from guanine (Margison and Santibanez-Koref, 2002; Kaina et al., 2007). If repair of O^6 MeG by MGMT is complete, other methylation lesions, notably *N3*-methyladenine and *N3*-methylguanine, can become biologically relevant (Gates, 2009). During base excision repair (BER), apurinic sites are formed that block replication. In addition, BER intermediates can become cytotoxic if they are not repaired efficiently as a result of impaired or exhausted BER. Thus, cells lacking DNA polymerase β are hypersensitive to methylating agents (Sobol et al., 1996) because of the accumulation of repair intermediates, which is executed by apoptosis (Ochs et al., 2002).

In this study, we determined whether NBN protects cells against the killing effect of S_N1 methylating agents. To this end, human lymphoblastoid cells and human fibroblasts derived from patients with NBS were used, and their response to temozolomide and MNNG was determined. The data show for the first time that homozygous mutation of *NBN* leads to sensitization of cells to these agents, with O^6 MeG being the main toxic lesion and *NBN* involved in the processing of O^6 MeG triggered DSBs. Unexpectedly, killing of *NBN* mutant cells occurred predominantly by the induction of necrosis, whereas in wild-type (wt) cells, apoptosis is the main mode of cell death. We also show that siRNA mediated down-regulation of NBN in melanoma cells renders them highly sensitive to temozolomide. The data identified Nibrin as a new player of cell resistance to S_N1 methylating agents, including chemotherapeutics such as temozolomide, procarbazine, and dacarbazine.

Materials and Methods

Cell Lines and Culture Conditions. In this study, three Epstein-Barr virus-immortalized human lymphoblastoid cell lines [95P466 (wt), 94P247 (NBN mt), and 94P126 (NBN mt)] (Krüger et al., 2007) and two simian virus 40-immortalized human fibroblast cell lines, wild type and mutated for NBN [GM637 (wt) and NBS-1LBI (NBN mt)] (Kraakman-van der Zwet et al., 1999), were used. The line NBS-1LBI was a generous gift from Dr. M. Zdzienicka (Ludwik Rydygier University of Medical Sciences, Bydgosz, Poland). The cell lines LB1-LXIN and LB1-NBS-1 are derivatives of NBS-1LBI stably transduced with an empty retroviral vector (LXIN) and a retroviral expression vector harboring the human NBN cDNA, respectively. The expression level of the NBN transgene is shown in Fig. 4A. The melanoma cell line D03 was described previously (Naumann et al., 2009). All cell lines were cultivated in RPMI medium containing 10 to 15% fetal calf serum, and penicillin/streptomycin (10 U/ml, 0.1 mg/ml) in a humid atmosphere with 7% CO_2 at 37°C.

Drugs and Drug Treatment. MNNG (Sigma-Aldrich, Munich, Germany) and temozolomide (4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo [4.3.0]nona-2,7,9-triene-9-carboxamide; Schering-Plough, Whitehouse Station, NJ) were prepared and used as described previously (Roos et al., 2009a). To deplete MGMT, cells were pretreated with 10 μ M O^6 -benzylguanine (Sigma-Aldrich, Munich, Germany) 1 h before drug treatment. MGMT was depleted with O^6 -benzylguanine in all experiments unless specifically stated.

Viability Assay. For measuring the viability of the cells after mutagen treatment, WST-1 proliferation reagent obtained from Roche (Mannheim, Germany) was used. The procedure was performed according to the manufacturer's protocol.

Apoptosis and Necrosis Determined by Flow Cytometry. The cytotoxicity of mutagen treatment was measured by annexin V/PI double-staining and flow cytometry analysis as described previously (Naumann et al., 2009).

Alkaline Comet Assay. Treated and untreated cells were harvested and fixed on microscope slides with low melting point agarose. Cells were then lysed in ice-cold alkaline lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium laurylsarcosinate, 1% Triton X-100, and 10% DMSO, pH 10) for 60 min and incubated in ice-cold electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH >13) for 20 min. Afterward, cells were electrophoresed for 15 min at 25 V and 300 mA at 4°C. Slides were then neutralized (0.4 M Tris, pH 7.5), washed once with deionized water and dehydrated with 100% ethanol. For microscopy (Microphot-FXA; Nikon, Düsseldorf, Germany), dried slides were stained with propidium iodide (50 μ g/ml). Comet sizes were determined with Komet v4.0 software (Kinetic Imaging, Liverpool, UK).

Preparation of Whole-Cell Protein Extracts for Western Blot Analysis and MGMT Activity Assay. Whole-cell protein extracts (Naumann et al., 2009) and protein extracts for MGMT activity assay (Preuss et al., 1995) were prepared as described previously. Protein concentration was determined by the Bradford method (Bradford, 1976). For investigation of fibroblast protein levels, an additional protocol was used. Cells were harvested by adding 95°C lysis buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, and 10% β -mercaptoethanol, pH 6.8) directly onto attached cells. DNA was fragmented by sonication (Sonifier cell disruptor B15; Branson, Danbury, CT).

Western Blot Analysis. The method used is based on that described by Renart et al. (1979). The following primary antibodies were used: anti-cleaved caspase-7 (Cell Signaling Technology, Danvers, MA), anti-PARP-1 (BD Transduction Laboratories, Franklin Lakes, NJ), anti-MGMT (raised in the laboratory of B.K.), anti-NBN (Novus Biologicals, Cambridge, UK), anti-MSH2 (Oncogene Science Biomarker Group, Cambridge, MA), anti-MSH6 (BD Transduction Laboratories), anti-MLH1 (Santa Cruz Biotechnology), anti-PMS2 (BD Pharmingen, Heidelberg, Germany), and anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz,

California). Horseradish-peroxidase-coupled secondary antibodies were also used (Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, UK). Proteins were visualized using a chemiluminescence detection system (GE Healthcare).

BER Assay. BER assays using synthetic oligonucleotides, purchased from Eurofins MWG Operon (Ebersberg, Germany), were performed as described previously (Parsons et al., 2005).

MGMT Activity Assay. The method is based on a radioactive assay (Myrnes et al., 1984) that measures the transfer of a tritium-labeled methyl group from the O⁶-position of guanine to the MGMT protein in the cell extract and was performed as described previously (Preuss et al., 1995).

Transfection of Melanoma Cells with siRNA against *NBN*. siRNA knockdown of *NBN* was accomplished by cotransfection of D03 melanoma cells with the expression vector (pSuper) harboring the siRNA targeted toward *NBN* and the pSV2neo plasmid for selection. The siRNA sequence used for *NBN* gene silencing was 5'-GGCGUGUCAGUUGAUGAAA-3' (Zhang et al., 2005). The Effectene transfection kit (QIAGEN, Hilden, Germany) was used. Approximately 0.9 μ g of pSupersiNBN and 0.1 μ g of pSV2neo were transfected, and cells were selected with 1 mg/ml G418 (Sigma-Aldrich) until clones appeared. G418-resistant clones were picked, expanded, and tested for *NBN* protein expression. Stably transfected clones were routinely cultured in medium containing 1 mg/ml G418, which was omitted during the experiments.

Statistics. Statistical analysis was performed using Prism software (GraphPad Software, San Diego, CA). For comparing differences between two populations, the unpaired *t* test was performed. For regression fits, the linear equation was used.

Results

NBN-Deficient Cells Are Sensitive to S_N1 Methylating Agents Because of the Induction of Apoptosis and Necrosis. To determine the role of nibrin in the DNA damage response provoked by methylating agents, adherent and

nonadherent growing human cell lines from patients with NBS were treated with increasing doses of MNNG or temozolomide for 72 h and sensitivity was determined using the WST-1 proliferation assay. *NBN* mutated lymphoblastoid cells (Fig. 1, A and C) and *NBN* mutated fibroblasts (Fig. 1, B and D) were more sensitive to MNNG and temozolomide throughout the dose range tested compared with the corresponding wt. It is noteworthy that in these survival assays, MGMT was depleted by O⁶-benzylguanine (O⁶BG) to eliminate differences in sensitivity between the cell lines that were due to different activity of MGMT (shown in Fig. 6C).

The WST assay provides a rough measure of cell sensitivity. To determine which mode of cell death is responsible for the increased toxicity observed in *NBN*-deficient cells, flow cytometric analysis of annexin V and PI double-stained cells was performed after MNNG treatment. This allowed for differentiation between apoptosis (annexin V-positive cells) and necrosis (annexin V- and PI-positive cells). As shown in Fig. 2A, lymphoblastoid cells from patients with NBS (94P247) do not display a higher level of apoptosis but show a strongly enhanced level of necrosis compared with the corresponding wt (95P466). In fibroblasts from patients with NBS (NBS-1LBI), both apoptosis and necrosis were strongly enhanced after MNNG treatment compared with the wt (GM637) (Fig. 2B). Thus, unexpectedly, in both cell systems, *NBN* mutated cells displayed a dramatic increase in the level of necrosis, which contrasts with previous experiments using other repair defective mutants (see *Discussion*). The total level of induced cell death was calculated by combining the frequencies of apoptosis and necrosis. The data show that *NBN* mutated lymphoblastoid (Fig. 2C) and fibroblast cells (Fig. 2D) are more sensitive to MNNG than the corresponding wt.

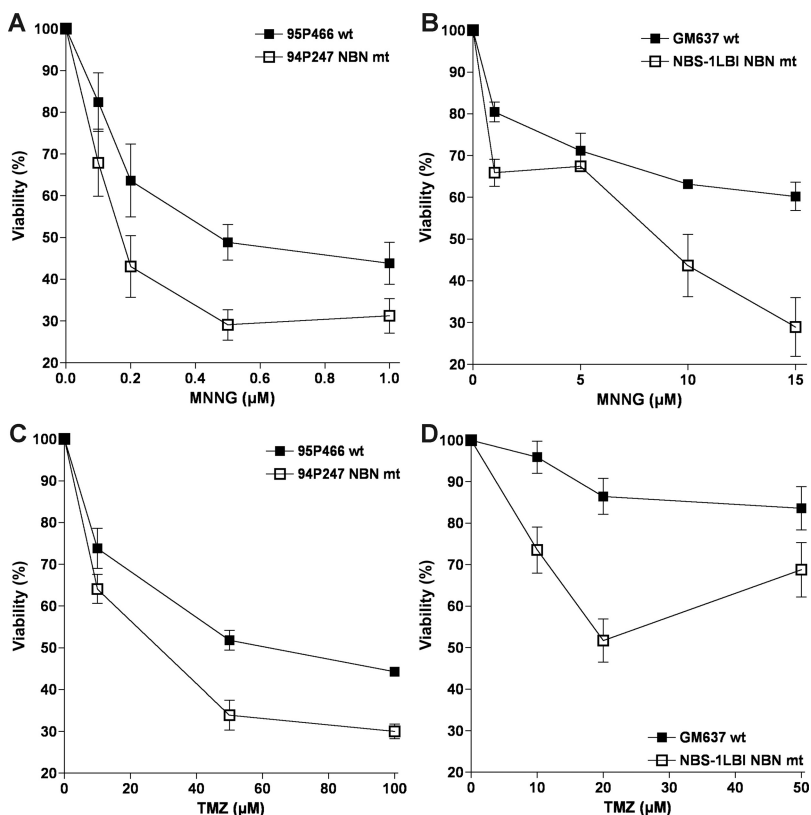


Fig. 1. Survival response of wt and *NBN* mutant cells treated with methylating agents. Cell viability was measured by WST-1 proliferation assay 72 h after treatment with indicated concentrations. Data are the mean of at least three independent experiments. A, lymphoblastoid cells after MNNG treatment. B, fibroblasts after MNNG treatment. C, lymphoblastoid cells after TMZ treatment. D, fibroblasts after TMZ treatment.

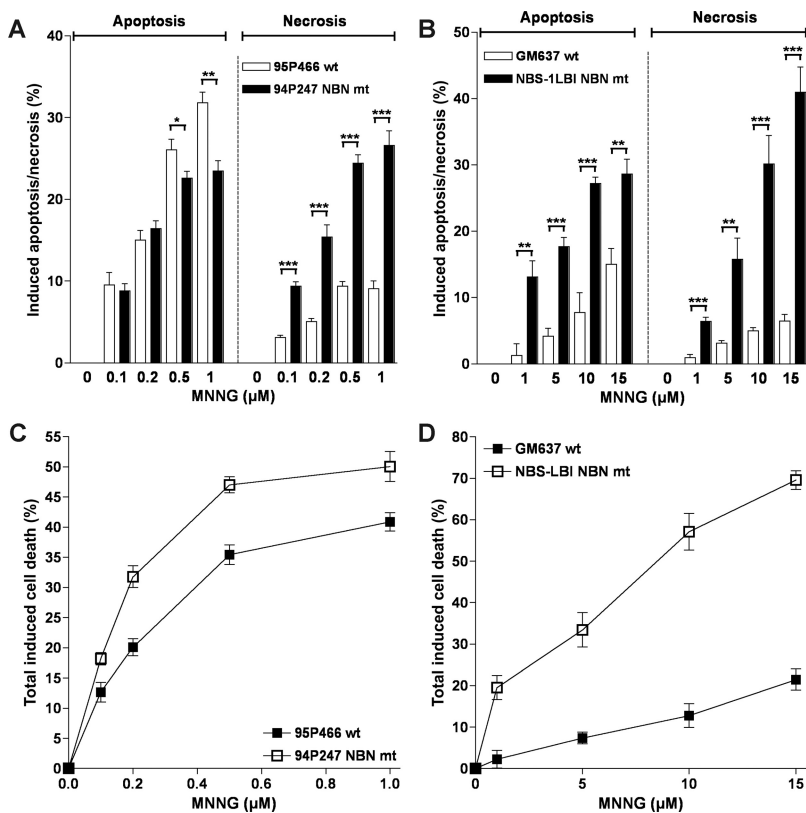


Fig. 2. Induced apoptosis, induced necrosis, and induced overall cell death upon treatment with MNNG. Annexin V/PI double-staining was performed 72 h after MNNG treatment with indicated concentrations. Data are the mean of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. A and B, induction of apoptosis (defined by annexin V-positive cells) and necrosis (defined by annexin V- and PI-positive cells) in lymphoblastoid cells and fibroblasts after MNNG treatment. C and D, induction of overall cell death (defined by combining the apoptotic and necrotic fraction) in lymphoblastoid cells and fibroblasts.

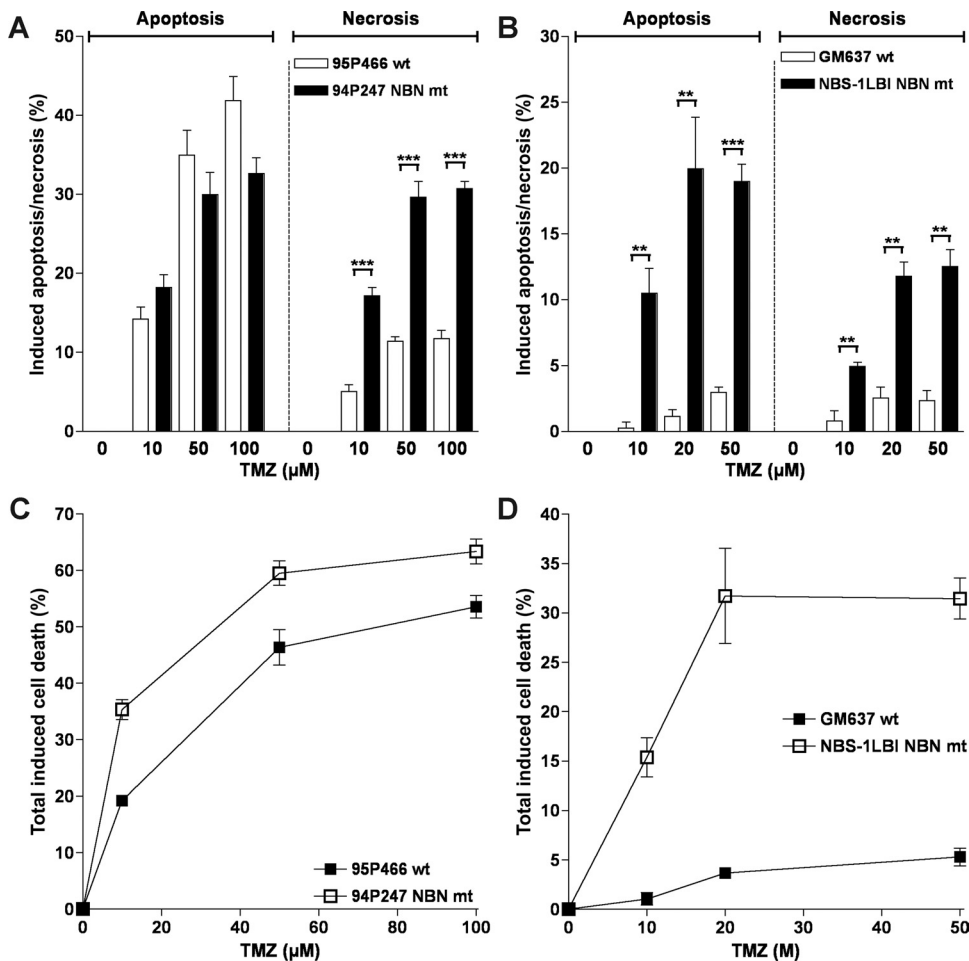


Fig. 3. Induced apoptosis, induced necrosis, and induced overall cell death upon treatment with TMZ. Annexin V/PI double-staining was performed 72 h after TMZ treatment with indicated concentrations. Data are the mean of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. A and B, induction of apoptosis (defined by annexin V-positive cells) and necrosis (defined by annexin V- and PI-positive cells) in lymphoblastoid cells and fibroblasts after TMZ treatment. C and D, induction of overall cell death (defined by combining the apoptotic and necrotic fraction) in lymphoblastoid cells and fibroblasts.

To further substantiate these findings, the lymphoblastoid and fibroblast cell pairs were treated with the anticancer drug temozolomide, which acts at the DNA level similar to the model S_N1 agent MNNG. Comparable results were obtained in that NBN-deficient cell lines were more sensitive to temozolomide than the corresponding wt (Fig. 3, A and B). Toxicity in *NBN* mutated lymphoblastoid cells was due to the induction of both apoptosis and necrosis, whereas in wt cells, with lower sensitivity, apoptosis was the major pathway (Fig. 3A). In fibroblasts from patients with NBS, both apoptosis and necrosis were induced at high level (Fig. 3B). TMZ led to more overall toxicity in lymphoblastoid (Fig. 3C) and fibroblast cells (Fig. 3D) than the corresponding wt.

To further verify the protective role of NBN, TMZ sensitivity was compared in an *NBN* mutated cell line complemented with full-length cDNA coding for human NBN and the *NBN* mutated cell line. The expression of the transgene is shown in Fig. 4A, inset. Complementation clearly protected cells from cell death induced by TMZ (Fig. 4A), indicating that the hypersensitivity of the cells was due to mutational inactivation of the *NBN* gene. Collectively, the data show that, if functional *NBN* is missing, lymphoblastoid cells and fibroblasts are hypersensitive to methylating agents and that increased cell death is executed both by apoptosis and necrosis.

Caspase-7 and PARP-1 Are Cleaved during Methylating Agent Induced Apoptosis in NBN Mutated Cells. In an effort to clarify whether the observed annexin V/PI double-staining data obtained in our experiments with methylating agents in *NBN* mutated cells truly reflects apoptosis, activation of pro-caspase-7 (an apoptosis executing caspase)

was assayed. Furthermore, cleavage of PARP-1, a downstream target of caspases, was analyzed. Using protein extracts of MNNG-exposed NBN defective lymphoblastoid cells and the corresponding wt, we observed that caspase-7 becomes activated by MNNG. This occurred at a slightly higher level in the wt than in the mutant cells (Fig. 4B), which is in line with the slightly higher frequency of apoptosis in the wt (Fig. 2A). Caspase-7 activation was paralleled by PARP-1 cleavage, a hallmark of apoptosis, which was also slightly higher in the wt (Fig. 4B). For fibroblasts, the NBN mutant showed strong activation of caspase-7, whereas no activation was observed in the wt. The same holds true for PARP-1 cleavage (Fig. 4B), which clearly corresponds to the high level of apoptosis upon MNNG treatment in NBN mutated fibroblasts (Fig. 2B). Overall, the data demonstrate that NBN mutated cells undergo apoptosis in response to methylating agent treatment, which is accompanied by caspase-7 activation and PARP-1 inactivation.

NBN Cells Do Not Exhibit Differences in BER. There are data suggesting that NBN may play a role in BER after exposure to MMS, which is a S_N2 methylating genotoxin (Sagan et al., 2009). To prove whether this is also the case for S_N1 methylating agents, wt fibroblasts and those from patients with NBS were exposed to 15 μ M MNNG and subjected to the alkaline comet assay at the indicated time points. After 1-h treatment, both wt and NBN mutated cells show a high amount of DNA single-strand breaks, which are indicative of BER intermediates (Fig. 5A). The kinetics of repair of these DNA breaks was similar in both wt cells and cells from patients with NBS, indicating that *NBN* mutated cells are able to repair BER intermediates. To substantiate this, a functional in vitro BER assay was performed with cell

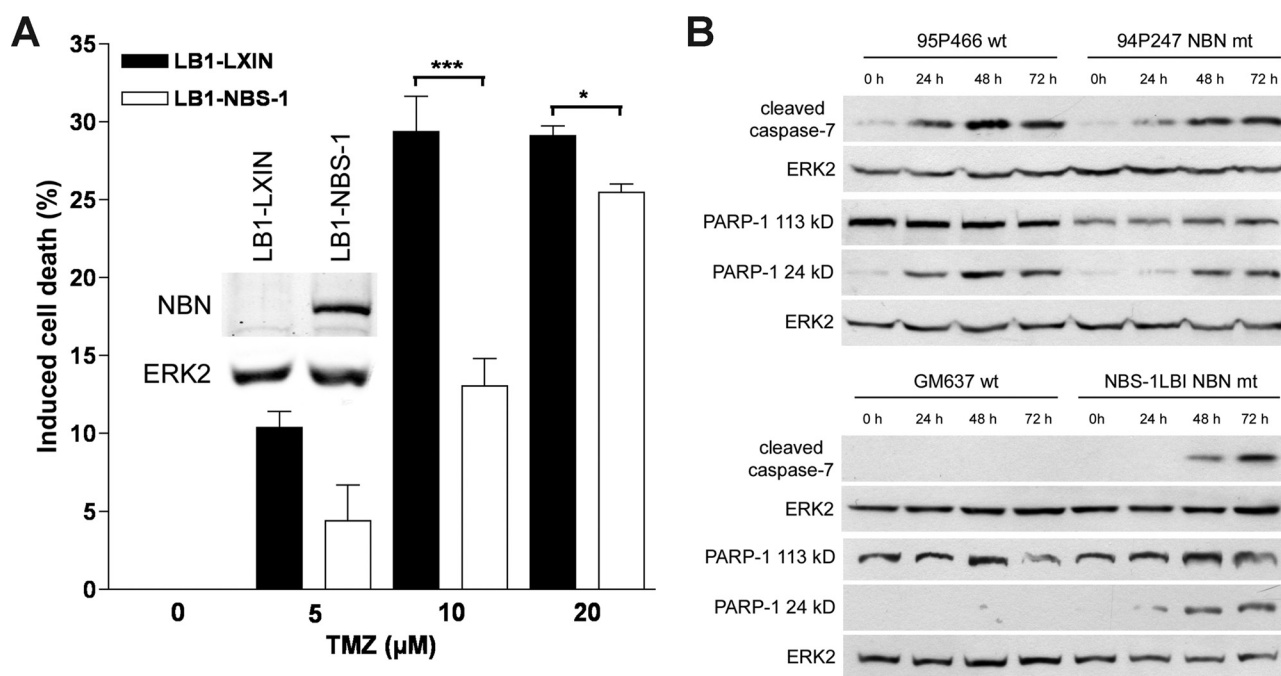


Fig. 4. Complementation of *NBN* mutant cell lines with *NBN* cDNA protects against TMZ and apoptosis-dependent proteolysis of caspase-7 and PARP-1 upon treatment with methylating agents. A, induction of overall cell death (calculated by combining induced apoptosis and necrosis) in NBN defective fibroblasts complemented with full-length NBN. Inset, Western blot analysis of NBN protein level in isogenic cell pair. Cells were treated with indicated concentrations of TMZ, and toxicity was determined by Annexin V/PI double-staining and fluorescence-activated cell sorting analysis. Data are the mean of at least three independent experiments. *, $p < 0.05$; ***, $p < 0.001$. B, activation of caspase-7 and PARP-1 cleavage in wild-type and NBN mutated cells after MNNG treatment (lymphoblastoid cells, 0.5 μ M; fibroblasts, 10 μ M). Whole-cell protein extracts were used for Western blot analysis. ERK2 was used as loading control.

extracts obtained from wt fibroblasts and those from patients with NBS. Cleavage of a double-stranded oligonucleotide substrate (39-mer) containing a single apurinic site by AP

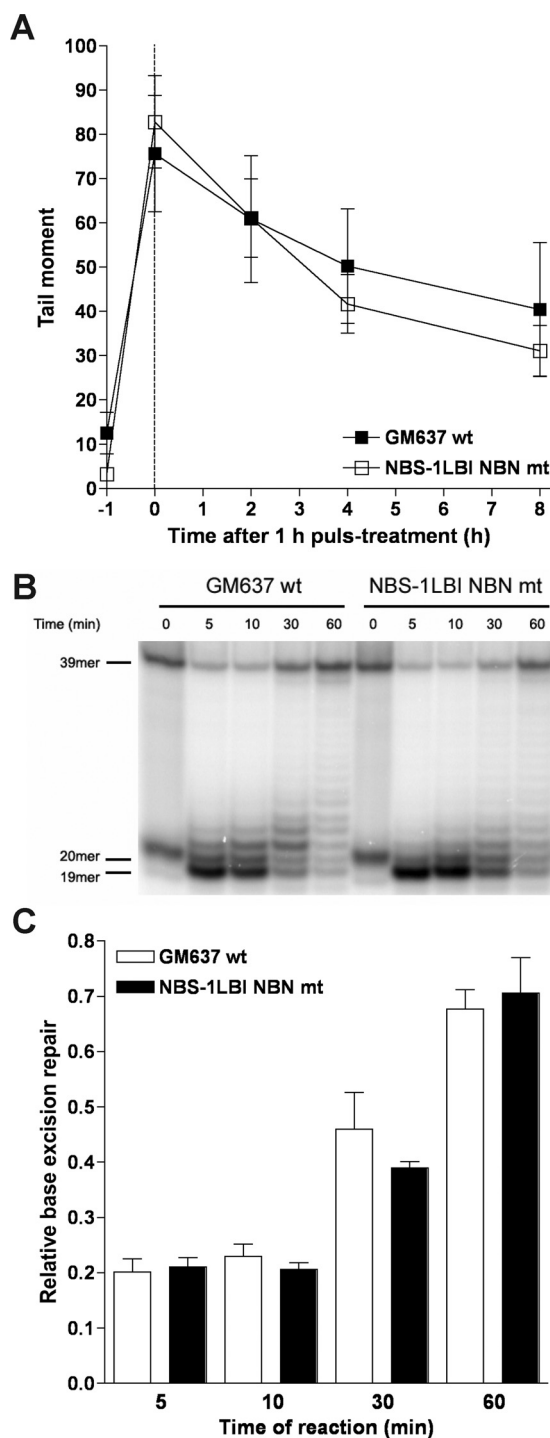


Fig. 5. Base excision repair in wild-type and NBN mutated fibroblasts. A, cells were treated with a pulse of 15 μ M MNNG for 1 h and DNA strand breaks were determined by the alkaline comet assay as a function of time after treatment. Data are the mean of three independent experiments. B, cleavage and reconstitution of a DNA-oligonucleotide containing a single apurinic site in the presence of whole-cell protein extract from wild-type and NBN mutated cells. Data of a representative experiment are shown. C, quantification of the 39-mer is shown. Relative BER capacity is expressed as fraction of reconstituted DNA-oligonucleotide compared with the zero time control. Data are the mean of three independent experiments.

endonuclease-1 results in a 19-mer labeled product and its further repair by DNA polymerase β and XRCC1-DNA ligase III complex restores the original full-length product. These repair events can clearly be visualized after gel electrophoresis and autoradiography of the gels (Fig. 5B). Quantification of the repaired DNA substrate revealed similar repair kinetics (Fig. 5C), supporting a similar repair capacity of NBN mutated and wt cells. Overall, the data shows that DNA repair by BER is not influenced by the absence of functional nibrin.

Increased Killing Response of NBN Mutant Cells Treated with Methylating Agents Is Due to O⁶-Methylguanine. The main determinants of methylating agent sensitivity are MGMT and MMR (Kaina et al., 2007). Therefore, we determined the MMR and MGMT status of the NBN wt and mutant cells used in this study. Concurrently, we confirmed the NBN status by Western blot. The wt cells 95P466 and GM637 clearly showed NBN protein expression, whereas no full-length NBN protein could be detected in the NBN mutated cell lines 94P247, 94P126, and NBS-1LBI (Fig. 6A). The MMR complex involved in the processing of O⁶MeG adducts comprises MSH2, MSH6, MLH1, and PMS2. As shown in Fig. 6B, Western blot analysis revealed that all the cell lines showed expression of the MMR proteins and are therefore not likely to be defective in MMR. We conclude that MMR does not account for the observed differences in sensitivity between wt and NBN mutated cells (Fig. 2 and 3).

The MGMT status of the cell lines was also determined. The lymphoblastoid wt cell line 95P466 displayed MGMT protein, whereas the NBN mutant line 94P247 did not. Neither wt fibroblasts nor those from patients with NBS expressed MGMT protein (Fig. 6B). These findings were confirmed by the analysis of MGMT activity determined in cell extracts (Fig. 6C). We searched for a cell line from patients with NBS that expresses MGMT, which was the case in 94P126 lymphoblastoid cells (Fig. 6, B and C). Both wt cells and cells from patients with NBS that express MGMT were completely resistant to MNNG (Fig. 6D), indicating that N-alkylations did not significantly contribute to the killing effect of MNNG in the dose range used. If MGMT was depleted by O⁶BG, cell death (executed by both apoptosis and necrosis) was promptly induced, which supports the conclusion that O⁶MeG is the critical killing lesion in both wt and NBN cells.

NBN Down-Modulation Causes Sensitization of Malignant Melanoma Cells to Temozolomide. It has recently been shown that high NBN expression in uveal malignant melanomas correlates with bad prognosis (Ehlers and Harbour, 2005). Because methylating agents are being used in the therapy of melanomas, we determined whether NBN in melanoma cells is involved in protection against temozolomide. To this end, NBN was stably knocked down in the melanoma cell line D03 (Fig. 7A for protein level). Any effect that MGMT might have on the differential sensitivity of the isogenic lines can be excluded, because the parental cells and the siRNA-transfected clones do not express MGMT (Fig. 7B). There was a clear increase in the killing response of D03siNBN13 cells, in which NBN was significantly down-regulated, after temozolomide treatment compared with the isogenic line, which pertained to the end points apoptosis (Fig. 7C) and necrosis (Fig. 7D). This is in line with data obtained with NBN mutants. When comparing the relative NBN protein level with the overall cytotoxicity induced by

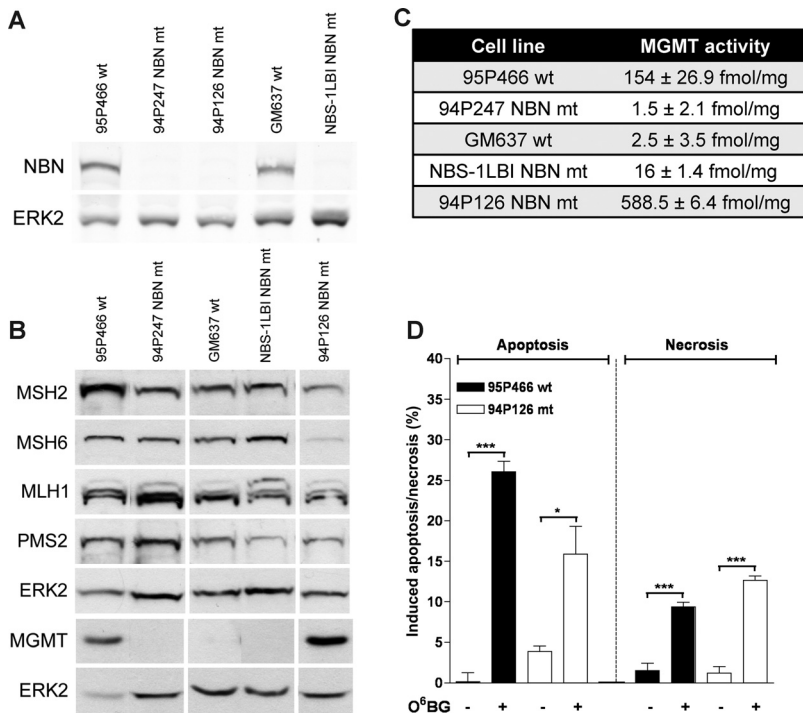


Fig. 6. NBN, mismatch repair and MGMT status of the cell lines, and effect of O⁶BG on MNNG-induced cell kill. A and B, NBN, mismatch repair proteins, and MGMT expression determined by Western blotting. Whole-cell protein extracts of untreated wt and NBN lymphoblastoid cells and fibroblasts were used. C, MGMT activity measured with radioactive MGMT activity assay. Data are the mean of two independent experiments. D, annexin V/PI double-staining of lymphoblastoid cells was performed 72 h after MNNG treatment (95P466, 0.5 μ M; 94P126, 5 μ M). The influence of O⁶BG pretreatment and presence of MGMT was determined. Because of its endogenous MGMT expression, 94P126 were used as NBN lymphoblastoid cells. Data are the mean of at least three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

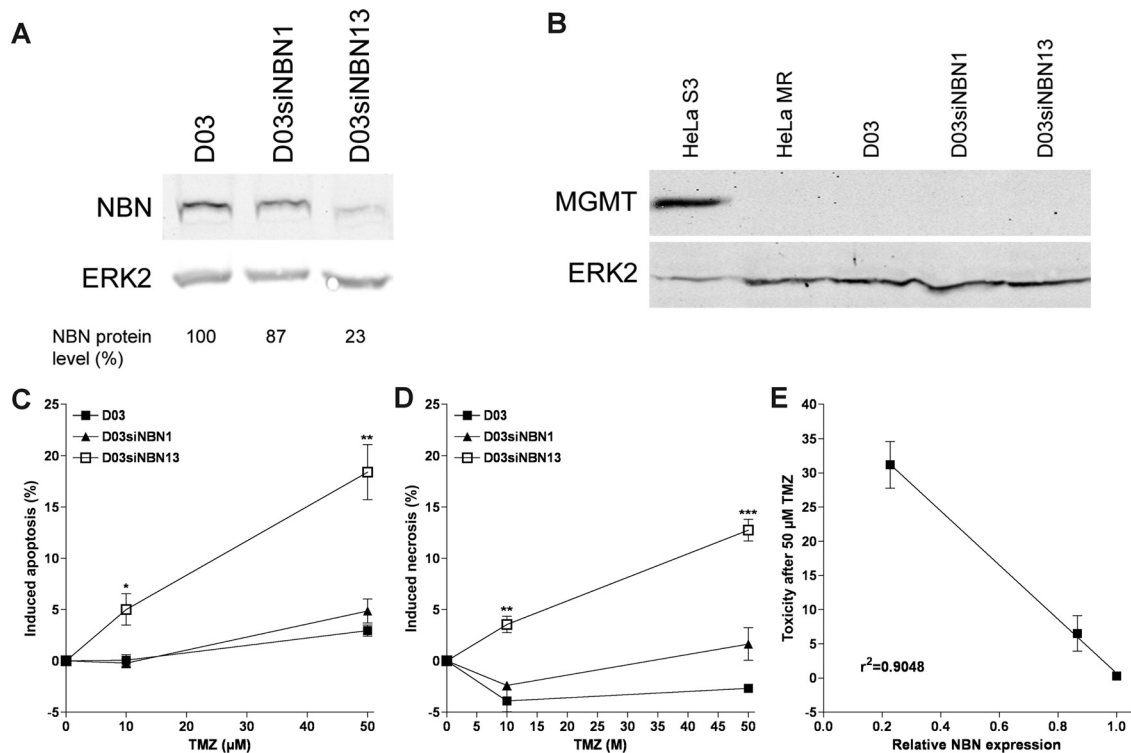


Fig. 7. NBN down-regulation sensitizes melanoma cells to temozolomide. A and B, Western blot analysis of NBN and MGMT protein in D03 and the stable NBN knockdown clones D03siNBN1 and D03siNBN13. The NBN protein expression level is shown on the bottom of the blot. NBN protein level of D03 was set to 100%, and the level of the stable knockdown clones was calculated as a percentage of D03 NBN protein and the loading as determined by ERK2. For MGMT analysis, protein extracts of HeLa S3 and HeLa MR served as positive and negative control, respectively. Apoptosis (C) and necrosis (D) induced by temozolomide in D03, D03siNBN13, and D03siNBN13 cells, determined 144 h after drug addition by annexin V/PI double-staining. Data are the mean of four independent experiments. P values were obtained by comparing data of D03 and D03siNBN13. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. E, toxicity induced by 50 μ M temozolomide as a function of the relative NBN protein level. The toxicity level was obtained by combining the apoptosis and necrosis results.

temozolomide, an inverse correlation was found (Fig. 7E), which is indicative of a dose effect of NBN on temozolomide resistance. The results strongly suggest NBN as a resistance marker for melanomas for which methylating drugs are applied in therapy.

Discussion

This work aimed to elucidate the role of NBN (NBS-1), which is a major component of the MRN complex, in the cellular defense against methylating agents. We used *NBN* mutated fibroblasts and lymphoblastoid cells as well as malignant melanoma cells knocked down for NBN and assessed their sensitivity to the model S_N1 agent MNNG and the similarly acting anticancer drug temozolomide. The data show that *NBN* mutant and knockdown cells are clearly more sensitive than the corresponding wt to the cytotoxic effect of methylating agents. The data revealed nibrin as a new player in the defense against S_N1 mutagens and a new factor in resistance to methylating anticancer drugs.

To assess whether the increased killing response of NBS patient cells is due to impaired processing of O^6 MeG-derived lesions, we compared MGMT-expressing versus MGMT-depleted *NBN* mutant cells. If the cells express functional MGMT, they became, in the dose range tested, nearly completely refractory to the killing effect of MNNG. Thus, MGMT strongly prevented cells from undergoing apoptosis and necrosis after methylation. This indicates that O^6 MeG is the critical primary lesion that triggers both apoptosis and necrosis in *NBN* mutant cells. This was surprising because N-alkylation lesions also contribute to cytotoxicity (Roos et al., 2009b) and, theoretically, NBN could be involved in this pathway. O^6 MeG has previously been identified as the critical killing lesion for S_N1 methylating agents that causes the formation of DSBs and evokes the DNA damage response in the second cell cycle after treatment (Roos et al., 2009a; Quiros et al., 2010). Because NBN represents an essential element in the trimeric MRN complex together with MRE11 and Rad50, which recognizes DSBs and helps ATM/ATR to become activated, it is reasonable to conclude that the increased sensitivity of *NBN* mutant cells to S_N1 agents is due to impaired recognition and/or repair of DSBs induced by O^6 MeG adducts. Because DSBs formed in response to O^6 MeG are repaired mainly by HR and not NHEJ (Roos et al., 2009a), it is reasonable to surmise that the protective role of NBN is related to HR. Alternatively, ATR-Chk1 downstream signaling to the cell-killing machinery might be involved, which, however, is more hypothetical.

Our data show that increased cell kill of *NBN* mutants was due to the induction of both apoptosis and necrosis. This was surprising because it is in contrast to our previous findings with other cell systems, including rodent cells (Ochs and Kaina, 2000), human lymphocytes (Roos et al., 2004), glioma (Roos et al., 2007), and malignant melanoma cells (Naumann et al., 2009), in which MNNG and temozolomide mostly induce apoptosis (contributing to ~90% cell death). The increased level of necrosis could be caused, in theory, by impaired BER that would result in strong PARP-1 activation and ATP depletion. Therefore, we examined BER in *NBN* mutant cells. In the alkaline comet assay, the repair kinetics of DNA single-strand breaks was similar in cells from patients with NBS and wt cells, indicating that after MNNG

treatment, BER intermediates are formed and repaired in a similar manner in wt and *NBN* mutant cells. This was substantiated in experiments using an in vitro BER assay, in which all steps of the BER pathway can be monitored (Dianov, 2003). Overall, the findings do not support a role for nibrin in BER, which contradicts a recent report (Sagan et al., 2009). The mechanism whereby *NBN* mt cells display an unusually high level of necrosis after O^6 MeG induction is currently under investigation.

Temozolomide is being used as first-line therapeutics in the treatment of the most severe form of brain tumor, GBM. It is noteworthy that an increased frequency of NBN mutations was recently demonstrated in GBM (Watanabe et al., 2009). On the basis of our data, it is pertinent to conclude that *NBN* mutations in GBM have an impact on the therapeutic response of the patients. Temozolomide and dacarbazine are also used in the treatment of malignant melanomas. In metastatic uveal melanomas, NBN expression was shown to be variable and a high expression level correlated with bad prognosis (Ehlers and Harbour, 2005). Here, we demonstrate that modulation of NBN expression by means of siRNA transfection had a clear impact on methylating agent sensitivity of malignant melanoma cells, which were previously shown to undergo apoptosis after TMZ treatment with O^6 MeG and DSBs being involved (Naumann et al., 2009). It is noteworthy that both apoptosis and necrosis were induced at higher level, similar to what we observed in *NBN* mutant cells. Overall, the data identify NBN as a novel therapeutic target in melanomas and presumably also other tumors, including GBM, for which methylating agents are being used as the "gold standard" in therapy (Middleton et al., 2000; Stupp et al., 2005). Because methylating agents are powerful carcinogens widely distributed in the environment and food, the data also bear implications for NBN as an important player in the protection against the toxicity brought about by these environmental genotoxins.

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