Overexpression of NBS1 Contributes to Transformation through the Activation of Phosphatidylinositol 3-Kinase/Akt*

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Nijmegen breakage syndrome (NBS) is a chromosomal instability syndrome associated with cancer predisposition, radiosensitivity, microcephaly, and growth retardation. The NBS gene product, NBS1 (p95) or nibrin, is a part of the hMre11 complex, a central player associated with double strand break repair. We previously demonstrated that c-Myc directly activates NBS1 expression. Here we have shown that constitutive expression of NBS1 in Rat1a and HeLa cells induces/enhances their transformation. Repression of endogenous NBS1 levels using short interference RNA reduces the transformation activity of two tumor cell lines. Increased NBS1 expression is observed in 40–52% of non-small cell lung carcinoma, hepatoma, and esophageal cancer samples. NBS1 overexpression stimulates phosphatidylinositol (PI) 3-kinase activity, leading to increased phosphorylation levels of Akt and its downstream targets such as glycogen synthase kinase 3β and mammalian target of rapamycin in different cell lines and tumor samples. Transformation induced by NBS1 overexpression can be inhibited by a PI3-kinase inhibitor (LY294002). Repression of endogenous Akt expression by short interference RNA decreases the transformation activity of Rat1a cells overexpressing NBS1. These results indicate that overexpression of NBS1 is an oncogenic event that contributes to transformation through the activation of PI3-kinase/Akt.

Nijmegen breakage syndrome (NBS) is an autosomal recessive hereditary disorder characterized by microcephaly, a “bird-like” facial appearance, growth retardation, immunodeficiency, radiosensitivity, chromosomal instability, and predisposition to tumor formation (1–3). The gene defective in NBS has been cloned, and the gene product, NBS1 (p95, nibrin), is a member of the DNA double strand break repair complex (hMre11 complex) including hMre11, hRad50, and NBS1 (1, 3). Increased radiation sensitivity and radiosensitive DNA synthesis of NBS fibroblasts are similar to the cellular features of AT (ataxia-telangiectasia) cells (2, 4), demonstrated by the recent results that ATM (ataxia-telangiectasia-mutated) protein phosphorylates NBS1 (5–7), linking these two proteins in the same pathway. NBS1 is a putative tumor suppressor gene as shown by the existence of NBS patients and some mutations discovered in different tumors (1, 2). However, NBS1 is expressed in highly proliferating tissues developmentally (8) and is located at sites of DNA synthesis through interaction with E2F (9). In addition, Mre11 complex is able to prevent double strand break accumulation during chromosomal DNA synthesis to ensure cell cycle progression (10). Nbs1 knock out in mouse embryonic stem cells shows the phenotype of diminished expansion of the inner cell mass of mutant blastocysts (Nbs1 null) (11, 12). Cellular proliferation defects are shown in Nbs1+/- mouse embryonic fibroblasts (13). Obviously, the roles of NBS1 are multiple, and some of them are still subject to intensive investigation.

Phosphatidylinositol (PI) 3-kinase is a major signaling component downstream of many growth factor receptor tyrosine kinases (14). Activation of PI3-kinase results in the phosphorylation of Akt (protein kinase B-PKB) to achieve its activation (15). Activated Akt then regulates a wide range of target proteins that control cell proliferation (e.g. glycogen synthase kinase-3β, GSK3β), survival (e.g. BAD), and cell growth (e.g. mammalian target of rapamycin, mTOR) (16). The PI3-kinase/Akt pathway obviously regulates processes that are critical for tumorigenesis (17), and altered expression or mutation of many components of this pathway has been implicated in human cancer (18, 19).

We previously demonstrated that NBS1 expression is directly activated by c-Myc oncoprotein (19). The c-Myc oncoprotein regulates diverse biological processes such as cell proliferation, growth, differentiation, and metabolism (20). Deregulated overexpression of c-myc occurs in a broad range of human cancers and is often associated with aggressive, poorly differentiated phenotypes (21). From the observation that overexpression of NBS1 increases cell proliferation rate and DNA synthesis (19), we speculated that overexpression of NBS1 might play a role in the process of tumorigenesis. Here we have shown that overexpression of NBS1 induces transformation activity. Increased NBS1 expression is observed in some types of human cancer. Overexpression of NBS1 stimulates phosphatidylinositol 3-kinase activity, leading to the activation of Akt and its downstream target proteins. These results demonstrate that overexpression of NBS1 is an oncogenic event that induces transformation through the activation of phosphatidylinositol 3-kinase/Akt.

**Experimental Procedures**

**Cell Lines and Plasmids—Lymphoblastoid cell lines (CB33, CBMycMax, CBMax) and RatNBS lines were previously described (19). The Rat1a and Saos2 cell lines were obtained from Dr. R. Dalla-Favera (Columbia University, New York). A549 cell line is a non-small cell lung...**

**References**

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5. The abbreviations used are: NBS, Nijmegen breakage syndrome; PI3-kinase, phosphatidylinositol 3-kinase; siRNA, short interference RNA; GSK3β, glycogen synthase kinase-3β; mTOR, mammalian target of rapamycin; ATM, ataxia-telangiectasis-mutated; NSCLC, non-small cell lung cancer; RT, reverse transcription; IHC, immunohistochemistry.
cancer (NSCLC) line. The pHeBOCMVNS plasmid was previously described (19). HeLaNBS and Saso2NBS cell lines were generated by transfecting the HeBOCMVNS plasmid into HeLa or Saso2 cells, and the cells were selected under G418 (250 µg/ml). Vector controls for all cell lines were generated by transfecting either pHeBOCMV, pSV2Neo, or pSV2Hygro (22). The pSUPERNBSi and pSUPERAktiR (knockdown rat Akt1) plasmids were generated by inserting the oligonucleotides 5'-GATCCCCTTGACATATGATGGGCTTCAAGAGGCTCATACTTATTTCTGAATTTTTGAAA-3' and 5'-GATCCCTTGGACATATTTTCAAGAGGATTTGCT-3' into the PSUPER plasmid provided by Dr. R. Agami (23). HeLaNBSi, A549NBSi, and RatNBSAkti cell lines were generated by transfecting pSV2Neo+pSUPERNBSi, pSV2Neo+pSUPERAktiR, or pSV2Hygro+pSUPERAktiR into HeLa, A549, or RatNBS cells, respectively, and the cells were selected under G418 (300 µg/ml) or Hygromycin (150 µg/ml). Wortmannin and LY294002 were purchased from Sigma.

Western Blot Analysis—60 µg of Rat1a or A549 cellular extracts or 100 µg of HeLa or Saso2 cellular extracts were loaded to an 8% SDS-PAGE gel and transferred to nitrocellulose filters. The filters were probed with an anti-NBS1 antibody (C-8580; Santa Cruz Biotechnology, Inc.), phospho-mTOR (Ser-2448) antibody (Signaling Technology, Inc.), phospho-GSK3 (Ser-9) antibody (Cell Signaling Technology, Inc.), Akt antibody (Cell Signaling Technology, Inc. or Cell Signaling Technology, Inc.), phospho-Akt (Ser-473) antibody (Cell Signaling Technology, Inc.), and TBP (TATA-binding protein internal control) used in RT-PCR were: NBS1, 5'-GAAATTGAGTTCCGCAGTTGTC-3'; TBP, 5'-GCGGAGCTCCACCTGCATAG-3'; G418, 5'-GAAATTGAGTTCCGCAGTTGTC-3'; c-Myc, 5'-GAAATTGAGTTCCGCAGTTGTC-3'; and 5'-GAAGGTTTGGAGAAGTATGAGCCTTCAAGAGGCTCATACTTATTTCTGAATTTTTGAAA-3' to the pSUPER plasmid provided by Dr. R. Agami (23). HeLaNBSi, A549NBSi, and RatNBSAkti cell lines were generated by transfecting pSV2Neo+pSUPERNBSi, pSV2Neo+pSUPERAktiR, or pSV2Hygro+pSUPERAktiR into HeLa, A549, or RatNBS cells, respectively, and the cells were selected under G418 (300 µg/ml) or Hygromycin (150 µg/ml). Wortmannin and LY294002 were purchased from Sigma.

Soft Agar Colony Assay—The stable clones were plated at three different cell densities (5 × 10^3, 10^4, 2 × 10^4, 2.5 × 10^4, 5 × 10^4) using standard assay conditions as mentioned (22) except that 15% fetal calf serum was used. Data shown here are representative of two or more experiments from independent cell cultures. Treatment with LY294002 was performed by putting LY294002 (50 µM) into both bottom and top agar.

Nude Mouse Tumorigenicity Assays and Drug Treatment—Tumorigenicity assays were performed as described (24). Five mice were used for injection of each clone. Animals were monitored at periodic intervals for the appearance of tumors up to 25 days after injection. Data shown here are representative of two experiments and using different clones.

RESULTS

Overexpression of NBS1 Induced/enhanced Transformation—To explore the role of NBS1 overexpression in the induction of transformation, we used soft agar and nude mice tumorigenicity assays to test whether overexpression of NBS1 could cause the transformation of Rat1a fibroblasts and form tumors. NBS1-overexpressing Rat1a clones (RatNBS) (19) were used, and the result showed that four different RatNBS clones overexpressing NBS1, but not control RatCMV clones, had acquired clonogenicity in soft agar at levels comparable with those displayed by c-Myc-transformed Rat1a cells (RatMyc) (Fig. 1A), roughly correlating with the amount of NBS1 detectable by immunoprecipitation-Western blot analysis (Ref. 19 and data not shown). Fig. 1B is a representative picture of soft agar colony assay. Injection of these RatNBS clones into nude mice caused rapid tumor growth and visible tumor formation compared with no tumor formation in the control RatNBS7 cells. The tumors generated from RatNBS cells were visible by day 10. The average size of the tumors was ~15 mm in diameter by day 13 (Fig. 1C), demonstrating the in vivo tumorigenicity of RatNBS cells. We also tested whether overexpression of NBS1 is capable of enhancing transformation in a cancer cell line by generating HeLa cells overexpressing NBS1. Fig. 1D shows the increased expression of NBS1 in three different HeLa clones (HeLaNBS) versus the control HeLa clone. The soft agar colony formation activity increased ~90–150% in these HeLaNBS clones compared with the control HeLa clone (data not shown), demonstrating the ability of NBS1 overexpression to enhance transformation activity in HeLa cells. Clonal populations of HeLa cells transfected with NBS1 expression vector also showed increased colony formation (>100% above control) compared with
HeLa cells transfected with control vector alone (data not shown). In addition, when these HeLaNBS clones were injected into nude mice, increased tumor volume was observed in these HeLaNBS clones (Fig. 1E). Fig. 1F is a representative picture taken from these nude mice at day 19. The HeLaNBS clones also had an increased cell proliferation rate and DNA synthesis compared with the control HeLa clone (data not shown). These results indicate that overexpression of NBS1 is capable of inducing/enhancing tumorigenicity of Rat1a/HeLa cells both in vitro and in vivo.

siRNA-mediated Repression of Endogenous NBS1 Expression Reduced the Transformation Activity of Two Tumor Cell Lines—We tested whether the levels of NBS1 expression correlated with transformation...
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activity by generating HeLa cells expressing short interference RNA (siRNA) to repress their endogenous NBS1 expression (23). Fig. 2A shows the three HeLaNBSi clones with decreased NBS1 protein levels versus the control HeLa clone. Soft agar colony formation activity decreased in these three HeLaNBSi clones versus the control HeLa clone (Fig. 2B). The levels of NBS1 roughly correlated with the levels of soft agar colony numbers. We also tested the in vivo tumorigenic activity of these HeLaNBSi clones by injecting these clones into nude mice. Fig. 2C shows the decreased tumor volume (~55% of control) of two HeLaNBSi clones versus the control HeLa clone. The NSCLC line A549 was also used to test this correlation. Two A549 clones expressing siRNA to repress endogenous NBS1 expression were generated (Fig. 2D). Fig. 2E shows the decrease (~40% of control) in soft agar colony numbers in the A549NBSi clones versus the control A549 clone. Nude mice tumorigenicity assays showed the decreased tumor volume (~45% of control) of these A549NBSi clones versus the control A549 clone (Fig. 2F). These results demonstrate the correlation between the levels of NBS1 expression and in vitro/in vivo transformation activity in two different tumor cell lines.

Increased NBS1 Expression Was Observed in a Certain Percentage of Some Different Human Cancer Samples—We wanted to test whether increased NBS1 expression occurs in human tumor samples. Twenty-nine pairs of human NSCLC samples (normal versus tumor tissues) were used to screen for NBS1 expression levels using RT-PCR analysis. Increased NBS1 expression was observed in 52% (15 of 29) of these lung cancer samples, indicating that increased NBS1 expression indeed occurred in human tumor samples. The RT-PCR results were confirmed using quantitative real-time PCR analysis (the fold of increase ranged from 2- to 6-fold). Fig. 3A and B, shows the pictures of RT-PCR and real-time PCR results in some representational cases. NBS1 protein levels were also examined in these pairs of lung cancer samples, and their levels showed corresponding increases in samples with increased NBS1 mRNA expression. Fig. 3C shows the increased NBS1 protein levels in these representative pairs of lung cancer samples. Screening for increased NBS1 expression in hepatoma and esophageal cancer also showed certain percentages (48 and 40%) of increased NBS1 expression using RT-PCR and quantitative real-time PCR analysis (Fig. 3D). These results demonstrate that increased NBS1 expression occurs in a certain percentage of three types of human cancer.

Overexpression of NBS1 Activated Akt and Led to the Activation of Its Downstream Target Proteins—To search for the signal transduction pathways that mediate transformation induced by NBS1 overexpression, we screened different signal transduction/apoptosis-related molecules for their contribution to transformation in NBS1-overexpressing cell lines. The panel includes extracellular signal-regulated kinase 1/2, Bcl-2, Bax, Akt/PKB, etc. The results of the screening showed that only Akt was activated by NBS1 overexpression. Western blot analysis using an anti-phospho-Akt antibody showed that Akt phosphorylation levels were increased in RatNBS clones (Fig. 4A). This result was confirmed in an osteosarcoma cell line, Saos2 cells overexpressing NBS1 (Fig. 4A). In addition, the A549 clones expressing siRNA (A549NBSi) to repress endogenous NBS1 expression showed decreased Akt phosphorylation levels (Fig. 4B), correlating the levels of NBS1 expression with the levels of Akt phosphorylation. To demonstrate the stimulation of downstream targets by activated Akt, we examined different Akt downstream targets. Among them, GSK3β and mTOR were shown to be hyperphosphorylated in NBS1 overexpression status in RatNBS and Saos2NBS cells (Fig. 4A). Their levels of phosphorylation (GSK3β and mTOR) also decreased in NBS1 knockdown status in A549NBSi cells (Fig. 4B). To confirm that activation of Akt by NBS1 overexpression also occurred in human tumor samples, immunohistochemistry analysis using anti-NBS1 and anti-phospho-Akt antibodies was performed in NSCLC samples. The results showed that 13 of 15 NSCLC samples overexpressing NBS1 had increased Akt phosphorylation levels (data not shown). Representational IHC results are shown in Fig. 4, C–E. These results demonstrate that the levels of NBS1 correlate with the phosphorylation levels of Akt and its downstream targets, GSK3β and mTOR, in different cell lines and human tumor samples.

Overexpression of NBS1 Stimulated PI3-Kinase Activity, Which Can Be Inhibited by PI3-Kinase-specific Inhibitors—To test whether the activation of Akt was induced by increased upstream PI3-kinase activity, we performed a PI3-kinase activity assay using RatCMV versus RatNBS.
cells (27). The result showed that PI3-kinase activity indeed increased (~90% above the control) in the RatNBS clone versus the control RatCMV clone (Fig. 5A). PI3-kinase activity in both clones can be inhibited by LY294002, a PI3-kinase-specific inhibitor. In addition, siRNA experiments to repress endogenous NBS1 levels in A549 cells (A549NBSi) caused a decrease in PI3-kinase activity (data not shown). These results demonstrate the stimulation of PI3-kinase activity by NBS1 overexpression. Both PI3-kinase inhibitors, LY294002 and wortmannin, also inhibited the Akt phosphorylation levels in the RatNBS clone (Fig. 5B), demonstrating the requirement for the stimulation of PI3-kinase, leading to activation of downstream Akt. We also tested whether LY294002 can inhibit the in vitro tumorigenicity of RatNBS clone using soft agar colony assays. Fig. 5C shows the almost complete inhibition of soft agar colony formation of RatNBS clone by LY294002. We performed experiments using LY294002 to treat nude mice injected with RatNBS cells to test the ability of LY294002 to inhibit RatNBS-induced tumors in vivo. Fig. 5D shows the inhibition (a decrease of ~65%) of RatNBS tumor growth by LY294002 compared with injection with solvent alone. To test that the shrinkage of tumors was not due to nonspecific toxicity caused by LY294002, immunohistochemistry analysis of tumor tissues from the mice treated without or with LY294002 was performed. The result showed that tumor tissue not receiving LY294002 still had strong phospho-Akt staining, whereas tumor tissue receiving treatment did not have phospho-Akt staining (Fig. 5E). This result demonstrated that treatment with LY294002 decreased the Akt phosphorylation levels in RatNBS cells in vivo. Drug inhibition experiments on in vitro and in vivo tumorigenesis of the RatNBS clone show the ability of a PI3-kinase-specific inhibitor (LY294002) to inhibit tumorigenesis of RatNBS cells.

Repression of Akt Expression by siRNA Decreased the Transformation Activity of Rat1a Cells Overexpressing NBS1—We tested whether activation of Akt is critical for transformation mediated by NBS1 overexpression. We used the siRNA approach to repress endogenous Akt expression in RatNBS cells. Fig. 6A shows decreased Akt expression in two different RatNBSAkti clones, expressing siRNA to repress endogenous Akt. Fig. 6B shows decreased soft agar colony formation activity in these two clones compared with the control RatNBS clone. Nude mice tumorigenicy assays showed decreased tumor volume (~30% of control) of the RatNBSAkti clones versus the control RatNBS clone (Fig. 6C). This result demonstrates that the transformed phenotypes mediated by overexpression of NBS1 in RatNBS cells could be inhibited by siRNA-mediated repression of endogenous Akt.

Activation of PI3-Kinase/Akt in c-Myc-overexpressing Lymphoblastoid Cells—To test whether overexpression of c-Myc will also activate PI3-kinase/Akt due to its direct activation of NBS1 expression, Western blot analysis was performed in lymphoblastoid cells overexpressing c-Myc and Max (CBMyc-Max) (19). Fig. 6D shows that indeed CBMyc-Max cells had increased levels of NBS1 and phosphorylated Akt compared with the control CB33 or CB33Max cells. In addition, PI3-kinase assay showed the increased PI3-kinase activity in CBMyc-Max cells (~35% above control) (Fig. 6E). These results demonstrate that c-Myc overexpression directly activates NBS1 expression (19), leading to subsequent downstream activation of the PI3-kinase/Akt pathway.

DISCUSSION

In this report, we have demonstrated from the following evidence that overexpression of NBS1 is a dominant oncogenic event: 1) overexpression of NBS1 in Rat1a and HeLa cells induces/enhances their transformation activity; 2) the siRNA approach to repress endogenous NBS1 expression decreases the transformation activity of two tumor cell lines; 3) increased NBS1 expression occurs in a certain percentage of some types of human cancer; and 4) overexpression of NBS1 activates the PI3-kinase/Akt pathway. NBS1, as many other DNA repair checkpoint proteins, was initially known as a putative tumor suppressor to guard the integrity of genome (1–3). NBS1 carries out its checkpoint functions when it is phosphorylated by ATM protein after ionizing radiation (5–7). However, on the basis of the results presented here, we propose that NBS1 is also an oncoprotein. NBS1 overexpression-induced tumorigenicy may not act through the disruption of the functional Mre11/Rad50/NBS1 (MRN) complex, given the result that NBS1 knockdown cells (HeLaNBSi and A549NBSi), which contained reduced functional MRN complex, did not show enhanced transformation (Fig. 2). On the contrary, the siRNA approach to repress endogenous hMre11 levels increased the clonogenicity activity of HeLa cells, suggesting that the roles of NBS1 versus hMre11 related to transformation activity may be different (data not shown). The induction of tumorigenicity by NBS1 overexpression may go through the activation of an oncogenic pathway (PI3-kinase/Akt in this report) or the repression of a
tumor suppressor, whereas mutation of NBS1 could also contribute to tumorigenesis through deficiency in DNA repair leading to genomic instability (1–3). However, deletions or mutations of NBS1 have never or infrequently been shown in some types of human cancer (28–31). We demonstrate that the incidence rate of increased NBS1 expression is higher than that of NBS1 mutation or deletion in some types of human cancer (Fig. 3D). The higher percentage (40–52%) of increased NBS1 expression in certain human tumor types (non-small cell lung carcinoma, hepatoma, and esophageal cancer) supports its role in promoting transformation/tumorigenesis. Recent studies to discover prognostic markers in uveal melanoma also show that NBS1 overexpression is a significant prognostic marker (32), which is consistent with our results. Increased NBS1 expression may be due to activation by c-Myc (19) or other mechanisms because a low percentage (~10%) of non-small cell lung cancer, hepatoma, and esophageal cancer showed c-Myc overexpression (33–35).

The proliferation-inducing function of NBS1 is suggested by the expression of NBS1 in highly proliferating tissues developmentally (8) and by the ability of Mre11 complex to prevent double strand break accumulation during chromosomal DNA synthesis to ensure cell cycle progression (10). In addition, the phenotypes of diminished expansion of the inner cell mass of mutant blastocysts (Nbs1 null) and cellular proliferation defects in Nbs1m/m mouse embryonic fibroblasts also support the role of NBS1 in cellular proliferation (11–13). NBS1 overexpression causes the activation of PI3-kinase/Akt, which induces transformation through increasing cell proliferation and promoting cell growth by phosphorylating its various downstream target proteins (GSK3β and mTOR in this report) (16). Repression of PI3-kinase/Akt pathway in NBS1-overexpressing cells by two different approaches (PI3-kinase-specific inhibitors and siRNA to repress endogenous Akt) establishes the connection between NBS1 and PI3-kinase/Akt pathway, although this result may not indicate that Akt is on an NBS1-induced signaling pathway directly. Activation of PI3-kinase/Akt by increased NBS1 expression argues against the contention that NBS1 overexpression is just an accompanying side event during cancer development. The molecular mechanisms of NBS1 overexpression-mediated activation of PI3-kinase/Akt remain to be determined. Overexpression of NBS1 may contribute to increased PI3-kinase/Akt activity in a certain percentage of non-small cell lung cancer cell lines and patient tumor samples (36–38). Increased Akt phosphorylation levels were also shown in our NSCLC samples with NBS1 overexpression (data not shown and Fig. 4, D–E). Overexpression of NBS1 may promote anchorage-independent growth and invasiveness in different types of tumors through activation of PI3-kinase/Akt pathway (39–42). Transformation of NBS1-overexpressing cell lines (RatNBS) can be inhibited by a PI3-kinase inhibitor (LY294002), providing therapeutic implications for tumors with increased NBS1 expression as demonstrated in the tumor samples examined (NSCLC, hepatoma, esophageal cancer) (Fig. 3) (18, 25, 38). Taken together, NBS1 is the first DNA double strand break repair gene whose overexpression is capable of inducing transformation and tumorigenesis through the activation of PI3-kinase/Akt, thereby further elucidating the molecular mechanism of tumorigenesis in certain types of human cancer.

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