Increased NBS1 Expression Is a Marker of Aggressive Head and Neck Cancer and Overexpression of NBS1 Contributes to Transformation

Muh-Hwa Yang,1,4 Wei-Chung Chiang,2 Teh-Ying Chou,3 Shyue-Yih Chang,5 Po-Min Chen,4 Shu-Chun Teng,6 and Kou-Juey Wu2

Abstract

Purpose: Head and neck squamous cell carcinoma (HNSCC) represents the sixth most frequent type of cancer worldwide. However, the molecular genetic alterations underlying its malignant behavior and progression are little known. We showed previously that c-MYC directly activates the expression of the DNA double-strand break repair gene NBS1, and NBS1 overexpression contributes to transformation. Here, we investigate the role of NBS1 overexpression in HNSCC.

Experimental Design: Immunohistochemistry analysis of NBS1 expression was done in 81 locally advanced HNSCC patients. Real-time PCR and Western blot analysis were used to confirm immunohistochemistry results. Human hypopharyngeal cancer cell lines (FADU) with overexpressing NBS1 (FADUNBS) or inducible short interference RNA to repress endogenous NBS1 (FADUNBSI) were generated by stable transfection. Soft agar clonogenicity assay was used to determine the transformation activity. Western blot analysis and phosphatidylinositol 3-kinase (PI3K) assay were done to evaluate the signaling pathways that were involved.

Results: NBS1 overexpression was identified in 45% of advanced HNSCC patients. It was an independent marker of poor prognosis. NBS1 expression levels correlated with the transformation activity of FADU clones and also correlated with the phosphorylation levels of Akt and its downstream target mammalian target of rapamycin (mTOR). PI3K activity was increased in NBS1-overexpressing FADU clones. NBS1 overexpression also correlated with increased Akt phosphorylation levels in tumor samples.

Conclusions: Increased NBS1 expression is a significant prognostic marker of advanced HNSCC, and the underlying mechanism may involve the activation of the PI3K/Akt pathway.

Carcinomas of the head and neck, including cancers originating from the oral cavity, oropharynx, hypopharynx, and larynx, represent the sixth most frequent type of cancer in the world (1). They are ranked the fifth most lethal cause for male cancer patients in Taiwan, responsible for ~1,560 deaths per year (2). More than 90% of head and neck cancers are squamous cell carcinoma (HNSCC; ref. 1), which are etiologically associated with betel nut (3), tobacco, and alcohol exposure (4). The treatment of early HNSCC includes surgical resection or radiotherapy. The organ preservation approach (induction chemotherapy followed by concurrent chemoradiotherapy) has recently been adopted to treat advanced HNSCC (5, 6). In spite of the improvement in diagnosis and management of HNSCC, long-term survival rates have improved only marginally over the past decade (1). The most commonly used prognostic markers of HNSCC are components of the staging system, including tumor stage and lymph node status (7–10). However, the accuracy of these clinical prognosticators is not as precise as desired (11). Molecular markers, such as ras, c-myc, epidermal growth factor receptor (EGFR), bel-2, and p53, have recently been reported to be associated with clinical status and patient prognosis (12–16). However, low incidence (such as c-myc and ras) and conflicting results from different reports (such as c-myc, bel-2, p53, and EGFR) limit their application in the clinical setting (1, 12–16). Developing new prognostic indicators to facilitate patient management is of the utmost importance.

Nijmegen breakage syndrome (NBS) is a chromosomal instability syndrome associated with cancer predisposition, radiosensitivity, and growth retardation (17–19). The NBS gene product, NBS1, is a part of the hMre11 complex, which plays a central role in the DNA double-strand break repair (18, 19) and prevents double-strand break accumulation during chromosome replication (20). NBS1 carries out its checkpoint functions when it is phosphorylated by the ataxia...
telangiectasia mutated protein (ATM) following ionizing radiation (21–23). However, rare or no mutations of NBS1 have been identified in certain types of human cancers (24–27). The possible proliferation-inducing function of NBS1 is supported by the phenotypes of diminished expansion of the inner cell mass of mutant blastocysts (Nbs1 null) and cellular proliferation defects in Nbs1−/− mouse embryonic fibroblasts (28–30). In addition, we showed previously that c-MYC, a dominant oncoprotein, directly activates NBS1 expression and that overexpression of NBS1 increases cell proliferation (31). Our recent results show that overexpression of NBS1 contributes to transformation through the activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway in cell lines and patient line (FADU). Increased NBS1 expression is correlated with the advanced HNSCC patients using immunohistochemistry, real-time PCR, and Western blot analysis. Increased NBS1 expression is a marker of aggressive HNSCC. Overexpression of NBS1 increases transformation in a head and neck cancer cell line (FADU). Increased NBS1 expression is correlated with the activation of PI3K/Akt pathway in cell lines and patient samples. These results show the oncogenic property of NBS1 overexpression and its role in the tumorigenesis and prognostic value of HNSCC.

**Patients and treatment.** From January 2000 to March 2003, 81 patients diagnosed as locally advanced squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and larynx (T3−T4NXM0) in Taipei Veterans General Hospital (Taipei, Taiwan) were enrolled in this study. Informed consent was obtained in writing before patient enrollment. This study has been approved by the Institutional Review Board of Taipei Veterans General Hospital. All patients were considered poor candidates for resection because surgery will cause significant functional impairment. Before the initiation of treatment, all patients received endoscopic biopsy for pathologic diagnosis. Study samples, including tumor and normal tissues, were obtained during diagnostic biopsy, and normal tissues were derived from neighboring site outside of the tumor. Both tumor and normal tissues for subsequent studies were confirmed by pathologists. The organ preservation protocol with two courses of induction chemotherapy (cisplatin + 5-fluorouracil) followed by concurrent chemoradiotherapy (including cisplatin + 5-fluorouracil and radiotherapy for primary tumor and neck up to 70 Gy in 35 fractions over 7 weeks) was applied to all patients. Evaluation of treatment response was done after induction chemoradiotherapy and 2 months after chemoradiotherapy. The response criteria were based on RECIST criteria as described previously (33). Patients then received follow-up examinations every 3 months over the first year, every 4 months over the second year, and every 6 months thereafter. The medium follow-up period was 27 months (range, 18-56 months). The clinical characteristics of 81 HNSCC patients are illustrated in Table 1.

**Immunohistochemistry.** Sections (6 μm thick) of tumor and neighboring normal tissue were cut from the frozen specimens for immunohistochemistry analysis. The samples were fixed in acetone, air-dried, and subsequently bathed in TBS solution (pH 7.6). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide. For NBS1 immunohistochemistry staining, a goat polyclonal anti-NBS1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at the dilution of 1:100 and incubated for 1 hour. The immunohistochemistry of phosphorylated Akt was done with an immunohistochemistry-specific anti-phosphorylated Akt (Ser473) antibody (Cell Signaling Technology, Inc., Beverly, MA) at the dilution of 1:50 and incubated at 4°C overnight. After reacting with a biotinylated secondary antibody for 30 minutes, antigen-antibody reactions were visualized using streptavidin-horseradish peroxidase conjugate (DAKO LSAB kit; DAKO, Los Angeles, CA), with 3-aminо-9-ethylcarbazole as the chromogen. All slides were counterstained with hematoxylin.

**Immunohistochemical scoring.** All immunohistochemistry staining was independently scored by two experienced pathologists. If there was discordance with immunohistochemistry scoring, a pathologist peer review will be done to consolidate the result into a final score. The pathologists scoring the immunohistochemistry were blinded to the clinical data. The interpretation was done in five high-power views for each slide, and 100 cells per view were counted for analysis. Because normal tissues may have moderate NBS1 nucleus staining (34), the NBS1 immunohistochemistry scoring was defined on an scale ranging from 0 to +++ as illustrated (Fig. 1): 0, no appreciable staining in cells; +, only nucleus staining and no detectable cytoplasmic staining; ++, appreciable nucleus staining with cytoplasmic staining in <25% of cells; +++, significant nucleus staining and strong cytoplasmic staining (>25% of cells with cytoplasmic staining, which obscures cytoplasm and/or nucleus). Only NBS1 level +++ staining was defined as increased NBS1 expression.

The scoring of phosphorylated Akt was as follows: 0, no appreciable staining in tumor cells; +, barely detectable staining in cytoplasm and/or nucleus compared with stromal elements; ++, readily appreciable brown staining distinctly marking tumor cell cytoplasm and/or nucleus; +++, dark brown staining in tumor cells completely obscuring cytoplasm and/or nucleus. The levels 0 and + were grouped as nonexpression, and levels ++ and +++ were grouped as overexpression according to the published report (35).

**RNA purification from patient samples and real-time PCR analysis.** We prepared RNA from fresh-frozen tumor and normal tissues using a RNeasy kit (Qiagen, Germany). Real-time PCR analysis was done using

| Table 1. Characteristics of 81 advanced HNSCC patients |
|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| **Variables** | **Patient, n (%)** |
| **Age** | | | | | | |
| Range | 38-74 | | | | | |
| Median | 48 | | | | | |
| **Gender** | | | | | | |
| Male | 77 (95.1) | | | | | |
| Female | 4 (4.9) | | | | | |
| **Site** | | | | | | |
| Hypopharynx | 28 (34.6) | | | | | |
| Larynx | 19 (23.5) | | | | | |
| Oropharynx | 11 (13.6) | | | | | |
| Oral cavity | 23 (28.4) | | | | | |
| **T stage** | | | | | | |
| T3 | 46 (56.8) | | | | | |
| T4 | 35 (43.2) | | | | | |
| **N stage** | | | | | | |
| N0 | 31 (38.3) | | | | | |
| N1-3 | 50 (61.7) | | | | | |
| **Response to chemotherapy** | | | | | | |
| Complete remission | 31 (38.1) | | | | | |
| Partial response | 20 (24.7) | | | | | |
| Stable disease | 6 (7.5) | | | | | |
| Progressive disease | 24 (29.7) | | | | | |
| Organ preservation rate | 53 (65.4) | | | | | |
A total RNA isolated from tumor and neighboring normal tissues. Quantitative real-time PCR was done in a PRISM ABI7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with the preset PCR program, and TBP (TATA box binding protein) was selected as an internal control (32). The primer sequences used in real-time PCR were NBS1

\[
\text{5'-ATGGAGGCCATATTTCCATGAC-3'}
\]

and

\[
\text{5'-CAAGCAGCAGAACTTGGAAG-3'}
\]

and TBP

\[
\text{5'-TGGTGGTGTTGTGAGAAGATGG-3'}
\]

and

\[
\text{5'-CGGTGGGCACTTACAGAAGG-3'}
\]

cDNA (0.1 μg) and primers (80 μmol/L) were used for reaction in 1/2 SYBR Green Mixture (ABI) in a total volume of 50 μL. Increased NBS1 expression in tumor tissue was defined as >100% increase above normal tissue.

Protein extraction and Western blot analysis. For extraction of proteins from tumor tissues, 500 mg tumor samples were ground with rotor in 500 μL cell lysis buffer [50 mmol/L Tris (pH 7.5), 30 mmol/L MgSO4, 8 mmol/L EDTA, 2 mmol/L DTT, and 2% Triton X-100] containing protease inhibitors. Cell lysates were clarified by centrifugation at 13,000 rpm, 4°C for 10 minutes. The same lysis buffer was used to extract proteins from cultured cells. The protein content was determined by Bradford method (Bio-Rad Laboratories, Hercules, CA).

For Western blot analysis, 50 μg protein extracts from FADU/CMV, FADUNBS, FADUNBSi, and FADUNBSim clones and tumor samples were loaded to 10% SDS-PAGE gels and transferred to nitrocellulose filters. The filters were probed with an anti-NBS1 antibody (NB100-143, Novus Biologicals, Inc., Littleton, CO), phosphorylated Akt (Ser473) antibody (Cell Signaling Technology), Akt antibody (Cell Signaling Technology), phosphorylated mammalian target of rapamycin (mTOR; Ser2448) antibody (Cell Signaling Technology), and an anti-β-actin antibody (Santa Cruz Biotechnology) or anti-γ-tubulin antibody (Santa Cruz Biotechnology) as loading control. Signals were developed using an enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, United Kingdom). Data are representative of two or more experiments from independent cell cultures.

Plasmids, transfection, and cell lines. The human hypopharyngeal cancer cell lines FADU was obtained from American Type Culture Collection (Manassas, VA). FADU cells were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS). The pHBOCMVNBS plasmid was constructed as described (31). FADUNBS cell lines were generated by transfecting the pHBOCMVNBS plasmid into FADU cells and selection under G418 (200 μg/mL). Vector control cell lines (FADUCMV) were generated by transfecting pHBOCMV into FADU cells.

The plasmids pSUPERIORNBSi and pSUPERIORNBSim were generated by inserting the oligonucleotide of

\[
\text{5'-GATCCCCTTCAGAATAGAGTATGAGCCTTCAAGAGAGGCTCATACTCTATTCTGAATTTTTGGAAA-3'}
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and the mismatched oligonucleotide of

\[
\text{5'-GATCCCCTTCAGAATATAGTATGAGCCTTCAAGAGAGGCTCATACTATATTCTGAATTTTTGGAAA-3'}
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into the pSUPERIOR plasmid, respectively (OligoEngine, Inc., Seattle, WA). To establish the inducible FADUNBSi or FADUNBSim clones, the pcDNA6/TR was transfected into FADU cells and selected under blasticidin (5 μg/mL). After selection of the clones expressing Tet repressor (FADUTetR), the pSUPERIORNBSi or pSUPERIORNBSim plasmid was transfected into FADUTetR cells and selected under puromycin (0.25 μg/mL). The repression of endogenous NBS1 expression was induced by adding doxycycline (1 μg/mL) to FADUNBSi or FADUNBSim cells and incubated for 24 hours.

Fig. 1. Immunohistochemical analysis of NBS1 protein expression and localization in the normal upper aerodigestive epithelium (A and C) and tumor tissues (B and D-F) of HNSCC patients. Magnification, ×400. There is no NBS1 protein expression (immunohistochemistry level 0; A) or the presence of nucleus staining only (immunohistochemistry level +; C) in normal epithelium. Negative NBS1 staining (immunohistochemistry level 0; B), nucleus staining only (immunohistochemistry level +; D), or nucleus + weak cytoplasmic staining (immunohistochemistry level ++; E) was categorized as NBS1 nonoverexpression. Nucleus + strong cytoplasmic staining (immunohistochemistry level +++; F) was categorized as increased NBS1 expression. Black arrows, nucleus staining; yellow arrows, cytoplasmic staining.

1 μg total RNA isolated from tumor and neighboring normal tissues. Quantitative real-time PCR was done in a PRISM ABI7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with the preset PCR program, and TBP (TATA box binding protein) was selected as an internal control (32). The primer sequences used in real-time PCR were NBS1

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Soft agar clonogenicity assay. Anchorage-independent growth of FDA1, FADUNBS, and FADUNBSi cells was examined by survival of colonies on soft agar as described previously, except that 15% FBS was used (36). Three different cell numbers (5 × 10^3, 10^4, and 2 × 10^5) were used. The dishes were incubated for 2 weeks and colonies were counted.

PI3K assay. PI3K assays were done as described (37). Briefly, the immunoprecipitate of FADUCMV, FADUNBS, FADUNBSi, or FADUNBSim extracts with an anti-p110α antibody (Santa Cruz Biotechnology) was washed thrice with TNTG buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol], washed twice with PI3K reaction buffer [20 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 0.5 mmol/L EGTA], and suspended in 50 μL PI3K reaction buffer containing 0.2 mg phosphadidylinositol (bovine liver; Avanti Polar Lipids, Inc., Alabaster, AL)/ml. The reactions were initiated by adding 5 μL MgCl2-ATP mixture (200 mmol/L MgCl2, 200 μmol/L ATP) containing 10 μCi [γ-32P]ATP to the reaction mixture and incubating the mixture at 25°C for 20 minutes. The reactions were terminated by adding 150 μL chloroform/methanol/11.6 N HCl (100:200:2). After addition of 120 μL chloroform to each sample, the organic phase was separated by centrifugation and washed twice with methanol/1 N HCl (1:1). After evaporation, the pellets were resuspended in 20 μL chloroform, spotted onto a silica gel plate, and developed in chloroform/methanol/28% ammonium hydroxide/water (43:38:5:7). The phosphorylated lipids were visualized by autoradiography. The intensity of phosphorylated lipids was measured by Phospholmage. Kinase activity was measured as percentage of lipids becoming phosphorylated by PI3K. Data are representative of results from two experiments using different FADUCMV and FADUNBS clones or repeated in FADUNBSi/FADUNBSim clones.

Statistical analysis. Statistical Package of Social Sciences software version 12.0 (SPSS, Inc., Chicago, IL) was used for statistical analysis. The Kaplan-Meier estimate was used for survival analysis, and the log-rank test was selected to compare the cumulative survival durations in different patient groups. The Cox’s proportional hazards model was applied in multivariate survival analysis to test independent prognostic factors. The level of statistical significance was set at 0.05 for all tests.

Results

Increased NBS1 expression in tumor tissues of advanced HNSCC patients. To investigate whether NBS1 overexpression may contribute to the tumorigenesis of HNSCC, immunohistochemistry analysis of NBS1 expression in 81 pairs of tumor and neighboring normal tissues of advanced HNSCC patients was done. In the normal tissue samples taken from these patients, 43 (53%) samples did not express NBS1 protein (immunohistochemistry level 0; Fig. 1A) and 38 (47%) samples showed nucleus staining only (immunohistochemistry level +; Fig. 1C). No NBS1 cytoplasmic staining could be detected in all normal tissues. For NBS1 expression in tumor tissues, 32 (40%) samples showed NBS1 negative staining or nucleus staining only (immunohistochemistry level 0 or +; Fig. 1B and D), 12 (15%) samples showed both nucleus and weak cytoplasmic staining (immunohistochemistry level ++; Fig. 1E), and 37 (45%) samples showed strong nucleus and cytoplasmic staining (immunohistochemistry level +++; Fig. 1F). These results indicated that increased NBS1 expression (immunohistochemistry level +++ in tumor tissues could be found in a significant proportion (45%) of advanced HNSCC patient samples (Fig. 2A).

To confirm the immunohistochemistry results of NBS1 expression, real-time PCR and Western blot analysis were done in representative tissue samples (NBS1 immunohistochemistry level +++ to correlate immunohistochemistry staining results with mRNA expression and protein levels. About 1- to 5-fold increase in NBS1 mRNA expression was observed in tumor tissues compared with the neighboring normal tissues using real-time PCR analysis (Fig. 2B). There is no increase or <1-fold increase in the samples of NBS1 immunohistochemistry staining 0 to + (data not shown). In addition, increased NBS1 protein levels in tumor tissues of HNSCC were also shown in representative pairs of HNSCC patient samples by Western blot analysis (Fig. 2C). These results showed the increased NBS1 expression in a significant percentage (45%) of advanced HNSCC patient samples.

Clinical significance of increased NBS1 expression in advanced HNSCC patients. To investigate whether increased NBS1 expression levels are associated with clinical outcome, Kaplan-Meier survival analysis was done to determine the prognostic significance of different NBS1 immunohistochemistry staining
levels. Patients with NBS1 immunohistochemistry level +++ staining were associated with a significantly shorter overall survival period compared with the immunohistochemistry level 0, +, and ++, respectively. Inset, \( P \) between each comparison. A, statistical comparison between NBS1 immunohistochemistry level +++ versus level 0 to ++ grouped together, which showed significantly shorter overall survival period in the NBS1 immunohistochemistry +++ patients (\( P < 0.001 \), log-rank). C and D, comparison of overall survival periods between NBS1 immunohistochemistry level +++ versus 0 to ++ in node-negative (\( N^- \); C) and node-positive (\( N^+ \); D) HNSCC cases.

**Fig. 3.** Prognostic significance of increased NBS1 expression in advanced HNSCC patients. A, individual comparison of prognostic value between NBS1 immunohistochemistry level +++ versus level 0, +, and ++, respectively. \( P \) between each comparison. B, statistical comparison between NBS1 immunohistochemistry level +++ versus level 0 to ++ grouped together, which showed significantly shorter overall survival period in the NBS1 immunohistochemistry +++ patients (\( P < 0.001 \), log-rank). C and D, comparison of overall survival periods between NBS1 immunohistochemistry level +++ versus 0 to ++ in node-negative (\( N^- \); C) and node-positive (\( N^+ \); D) HNSCC cases.

**Table 2.** Univariate survival analysis

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<th>Variables</th>
<th>Median overall survival (mo)</th>
<th>( P ) (log-rank)</th>
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</tr>
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<td>( \geq 50 )</td>
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<tr>
<td>Female</td>
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<td>Primary tumor site</td>
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<td></td>
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<tr>
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<td>Larynx</td>
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<td></td>
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<td>( T_3 )</td>
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</tr>
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<td>( T_4 )</td>
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<td>( N_1 )</td>
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<tr>
<td>Yes</td>
<td>15.4</td>
<td>&lt;0.001</td>
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<tr>
<td>No</td>
<td>—*</td>
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*Median survival was not reached.
Table 3. Multivariate survival analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (&lt;50 vs ≥50)</td>
<td>1.81 (1.12-2.76)</td>
<td>0.121</td>
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<tr>
<td>N stage (N1-3 vs N0)</td>
<td>3.38 (1.56-6.11)</td>
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<tr>
<td>NBS overexpression (yes vs no)</td>
<td>2.72 (1.39-3.52)</td>
<td>0.030</td>
</tr>
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NBS1 overexpression increased the transformation activity of HNSCC cells. To delineate the role of increased NBS1 expression in the tumorigenesis of HNSCC, the NBS1 expression vector (HeBOCMVNBS1; ref. 31) was stably transfected into a human hypopharyngeal cancer cell line (FADU) to establish NBS1-overexpressing clones (FADUNBS). Increased NBS1 expression could be identified in three FADUNBS stable clones compared with the vector (HeBOCMV)–transfected FADU clones (Fig. 4A). Soft agar clonogenicity assay showed the significantly increased colony formation activity (2.5- to 3-fold of control) in the FADUNBS clones compared with the vector control FADUCMV clones (Fig. 4B). Figure 4C showed the representative picture of the FADUCMV versus the FADUNBS clone. Clonal populations of NBS1-overexpressing FADU cells also showed increased colony formation activity (3-fold) compared with the vector control transfected FADU cells (data not shown). These results indicated that NBS1 overexpression increased the transformation activity of FADU cells. To further correlate NBS1 expression levels with the transformation activity of HNSCC cells, we constructed an inducible NBS1 knockdown FADUNBSi cell line in which endogenous NBS1 expression was repressed by doxycycline-inducible short interference RNA (siRNA) against NBS1 mRNA. Figure 4D showed that endogenous NBS1 expression of the FADUNBSi clone was successfully repressed (~50% of preinduction levels) by inducible siRNA. Clonogenicity assay of FADUNBSi cells before and after doxycycline treatment showed the decreased transformation activity (~55% of control) in the FADUNBSi clone following doxycycline induction (Fig. 4E). These results showed the correlation between NBS1 expression levels and transformation activity of FADU cells.

Correlation between NBS1 overexpression and the activation of PI3K/Akt pathway in HNSCC cell lines and patient samples. Our recent results show that overexpression of NBS1 contributes to transformation through the activation of PI3K/Akt (32). To show whether a similar mechanism was present in HNSCC cells overexpressing NBS1, Western blot analysis was done. The results showed that Akt phosphorylation levels increased in the FADUNBS clones (Fig. 5A), and decreased Akt phosphorylation levels were observed in the FADUNBSi clone following doxycycline induction (Fig. 5B). To show the activation of Akt pathway by NBS1 overexpression, we screened different Akt downstream targets. Among them, mTOR, whose phosphorylation regulates protein synthesis to increase cell growth (38), was hyperphosphorylated in FADUNBS cells (Fig. 5A). Decreased mTOR phosphorylation levels were also identified in the FADUNBSi clone following doxycycline induction (Fig. 5B). The control experiment using inducible mismatched siRNA to repress endogenous NBS1 expression in FADUNBSim clones undergoing doxycycline

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**Fig. 4. NBS1 overexpression increased the transformation activity of FADU cells.**

A, Western blot analysis showed the protein levels of NBS1 in the FADUCMV and FADUNBS clones. Actin was used as a control for protein loading. B, Soft agar colony formation assay was done in the FADUCMV and FADUNBS clones. C, a representative picture of clonogenicity assay (FADUCMV4 versus FADUNBS6) using 5 × 10³ cells in each dish. D, Western blot analysis of the FADU vector control clone and the FADUNBSi clone following doxycycline (Dox) treatment to induce siRNA and repress endogenous NBS1 expression. E, soft agar colony formation assay in the FADU vector control clone and the FADUNBSi clone without or with doxycycline treatment. *, P < 0.05, statistical significance between without and with doxycycline treatment in the FADUNBSi clone.
induction showed no decrease in the levels of NBS1 expression and phosphorylated Akt (Fig. 5C), showing the specificity of siRNA in FADUNBSi clones following doxycycline induction. To test whether the activation of Akt in the FADUNBS clone was induced by increased upstream PI3K activity, we did the PI3K activity assay in FADUNBS versus FADUCMV cells as well as FADU control versus FADUNBSi/FADUNBSim cells before and after doxycycline induction. The result showed that the FADUNBS clone had a 2-fold increase in PI3K activity compared with the FADUCMV clone (Fig. 5D). Repression of endogenous NBS1 expression in FADUNBSi cells caused a 64% decrease in PI3K activity compared with no decrease in PI3K activity in FADUNBSim cells following doxycycline treatment (Fig. 5E). These results showed that NBS1 overexpression correlated with the activation of PI3K/Akt pathway.

To confirm that the activation of PI3K/Akt pathway by NBS1 overexpression could occur in HNSCC patient samples, immunohistochemistry staining of phosphorylated Akt was done in 20 NBS1 immunohistochemistry level +++ staining patient samples, immunohistochemistry staining of phosphorylated Akt was done in 20 NBS1 immunohistochemistry level +++ and 20 immunohistochemistry level 0 to ++ patient samples. Predominantly cytoplasmic staining of phosphorylated Akt (level ++ to +++ as defined in Materials and Methods; ref. 35) could be detected in 18 of 20 (90%) NBS1 immunohistochemistry level +++ patient samples (Fig. 6A and B). On the contrary, level 0 to + staining of phosphorylated Akt was shown in 20 of 20 (100%) NBS1 immunohistochemistry level 0 to ++ patient samples (Fig. 6C and D). Western blot analysis confirmed the activation of Akt and its downstream target mTOR in representative HNSCC patient samples (Fig. 6E). These results showed that activation of Akt by NBS1 overexpression indeed occurred in HNSCC patient samples.

**Discussion**

In this report, we showed that increased NBS1 expression (NBS1 immunohistochemistry level +++ staining) occurred in a significant proportion (45%) of advanced HNSCC patients and was a significant prognostic marker of advanced HNSCC. NBS1, as with many other DNA repair checkpoint proteins, was initially known to guard the integrity of the genome (17–19). However, we propose that NBS1 may act as an oncoprotein and participate in the tumorigenesis of HNSCC due to the following reasons: (a) increased NBS1 expression is identified in 45% of advanced HNSCC patients and is associated with a poor prognosis; (b) overexpression of NBS1 increases the transformation activity of HNSCC cells, and siRNA approach to repress endogenous NBS1 expression decreases their transformation activity; and (c) NBS1 overexpression correlates with the activation of PI3K/Akt pathway. Our recent results showed that overexpression of NBS1 contributes to transformation through the activation of PI3K/Akt pathway. Recent studies to identify prognostic markers in uveal melanoma also showed that NBS1 overexpression is a significant prognostic marker (39), which is consistent with the results of this report.

Increased phosphorylation levels of Akt and its downstream targets have been identified in different types of human cancer (38, 40, 41), and the PI3K/Akt pathway is regarded as one of the most important oncogenic pathways in human cancers (42). PI3K-initiated pathway is the most frequently altered in HNSCC (43) and activation of Akt is shown to be a frequent event in HNSCC (44). Overexpression of eIF4E with the activation of Akt/mTOR pathway...
was also noted in the surgical margins of HNSCC (45). Activation of Akt was constantly associated with disease progression in different types of cancer (35, 41, 44). In this article, increased Akt phosphorylation levels could be identified in most HNSCC patients with increased NBS1 expression (Fig. 6). The proliferation-inducing function of NBS1 is suggested by the expression of NBS1 in highly proliferating tissues developmentally (46) and by the ability of Mre11 complex to prevent double-strand break accumulation during chromosomal DNA synthesis to ensure cell cycle progression (20). 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 supported the role of NBS1 in cellular proliferation (28–30). Overexpression of NBS1 causes the activation of PI3K/Akt, which induces transformation through increase of cell proliferation, promotion of cell growth, and inhibition of apoptosis by phosphorylating its various downstream target proteins (mTOR in this article; ref. 38). Activation of PI3K/Akt by increased NBS1 expression argues against the contention that NBS1 overexpression is just an accompanying side event during HNSCC development. Preliminary results showed the coimmunoprecipitation of NBS1 and one of the PI3K subunits, suggesting their direct interaction,7 which may lead to the activation of PI3K. Increased NBS1 expression may be due to the activation of c-MYC as we reported previously (31) or the involvement of other mechanisms because a low percentage (~10%) of HNSCC showed c-MYC overexpression (13).

In conclusion, our results show that overexpression of the DNA double-strand break repair gene NBS1 is capable of contributing to transformation and tumorigenesis possibly through the activation of PI3K/Akt, thereby further elucidating the molecular mechanism of tumorigenesis in HNSCC. Increased NBS1 expression is an independent prognostic marker of tumor progression in advanced HNSCC. This discovery provides valuable information for the identification and characterization of a novel molecular marker and a target for future therapeutic purposes in HNSCC.

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Increased NBS1 Expression Is a Marker of Aggressive Head and Neck Cancer and Overexpression of NBS1 Contributes to Transformation

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