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Identification of increased NBS1 expression as a prognostic marker of squamous cell carcinoma of the oral cavity

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Oral squamous cell carcinoma (OSCC) is one of the most prevalent cancers worldwide; however, accurate molecular markers to predict its prognosis are still limited. We previously demonstrated that overexpression of the DNA double-strand break repair protein NBS1 is a prognostic marker of advanced head and neck squamous cell carcinoma (HNSCC). Therefore, we aimed to investigate the feasibility of using NBS1 as a biomarker in OSCC. In this study, we enrolled 148 OSCC for immunohistochemical (IHC) and clinical analysis. Data from 58 advanced non-oral-cavity HNSCC (NO-HNSCC) cases were also included for comparison due to the biological and clinical discrepancy between OSCC and HNSCC originated from the other sites (e.g. pharynx or larynx). First, we validated the NBS1 IHC results by real-time RT-PCR analysis, and an excellent correlation between the results of these two assays confirmed the reliability and robustness of IHC procedures and interpretation. NBS1 overexpression was an independent prognostic marker in both OSCC and NO-HNSCC cases. In OSCC, the prognostic significance of NBS1 was shown regardless of T stage and lymph node status. Increased NBS1 expression correlated with advanced T stage and recurrence/metastasis. NBS1 overexpression correlated with the phosphorylation levels of Akt and its downstream target mammalian target of rapamycin (mTOR). These results clearly illustrate the expression profile of NBS1 in OSCC and NO-HNSCC, and highlight the role of NBS1 in HNSCC irrespective of the primary sites. It also indicates the practicability of application of NBS1 as a marker in OSCC. (*Cancer Sci* 2010; 101: 1029–1037)

Carcinomas of the oral cavity, including cancer originating from the tongue, buccal mucosa, gingiva, hard palate, and mouth floor, are among the 10 most common cancers in the world with the trend of increasing incidence.[1,2] The most common type of oral cancer is squamous cell carcinoma (OSCC), which accounts for more than 90% of oral malignancies.[1] The increasing incidence and dismal outcome of OSCC has become a public health major problem in Taiwan: habitual consumption of betel nuts results in a high prevalence of OSCC in Taiwan;[3–5] furthermore, most OSCC patients present with locoregional disease at diagnosis, and it ranks as the fourth most frequent cause of death among male cancer patients in Taiwan.[6]

The difference between OSCC and non-oral-cavity head and neck squamous cell carcinoma (NO-HNSCC), for example oropharyngeal, hypopharyngeal or laryngeal cancers, has been reported in several aspects. First, most OSCCs result from habitual consumption of betel nut or tobacco,[3–5,7] but smoking is one of the most important attributable factors for laryngeal cancer.[8] Furthermore, the pattern of developing second primary malignancies (SPM) in tongue cancer and laryngeal cancer is different. In tongue cancer patients, most of the SPMs appear in the oral cavity; in contrast, the lung and larynx are the most common sites of SPM for laryngeal cancer patients.[9] The treatment strategy is also different between OSCC and NO-HNSCC: although the effectiveness of chemoradiotherapy has been confirmed in pharyngeal and laryngeal cancers,[10,11] surgery remains the mainstay of treatment for advanced OSCC due to its poor response to chemotherapy.[12] In comparison with other sites of HNSCC, OSCC is also associated with a worse prognosis. The overall 5-year survival rate of OSCC patients is the second lowest among HNSCCs originated from different sites.[13] Chen *et al.* also demonstrated a worse outcome of tongue and mouth cancers compared with other sites of HNSCC.[14] Collectively, OSCC is different from NO-HNSCC in the etiology, patterns of SEM, treatment policy, and prognosis. Therefore, re-evaluation and validation of the findings in HNSCC is mandatory before extending to OSCC.

The dismal outcome of OSCC has remained unchanged during the past two decades despite significant advances in the diagnostic and therapeutic management of OSCC patients.[1,2,15] At present, positive lymph node involvement is the most decisive factor for the prognosis of OSCC patients;[16] however, its accuracy is not as precise as desired.[17] Molecular markers such as epidermal growth factor receptor, cyclin D1, and p53 have been reported to be important in OSCC. However, lack of evidence on prognostic value or conflicting results from different reports limit their application in the clinical setting.[1,18–21] Identification of molecular markers that can pinpoint patients with biologically aggressive tumors will be of the utmost importance for effective management of OSCC patients. Developing new markers that are easy to perform in clinical practice is also important.

Nijmegen breakage syndrome (NBS) is a chromosomal-instability syndrome associated with microcephaly, immunodeficiency, cancer predisposition, radiosensitivity, and growth retardation.[22–24] The product of the defective gene in NBS (the *NBS* gene), NBS1 (p95, nibrin), is a member of the DNA double-strand break (DSB) repair complex (hMre11 complex).[23,24] NBS1 carries out its checkpoint functions when it is phosphorylated by the ATM (ataxia-telangiectasia mutated) protein following ionizing radiation;[25–27] however, rare or no mutations of NBS1 have been identified in certain types of human cancer.[28–30] In addition, NBS1 is expressed in highly proliferating tissues during the developmental process.[31] We previously demonstrated that the oncoprotein c-MYC directly activates *NBS1* expression.[32] Overexpression of NBS1 contributes to

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transformation through the activation of PI3-kinase/Akt.[33,34] Increased NBS1 expression is present in 45% of advanced HNSCC patients receiving non-surgical treatment and is associated with a worse prognosis.[35] Overexpression of NBS1 induces the epithelial–mesenchymal transition (EMT), a pivotal mechanism of cancer metastasis, and is correlated with metastasis of HNSCC.[36] These results suggest the critical role of NBS1 overexpression in the progression and metastasis of squamous cell carcinomas of the upper aerodigestive tracts.

In this report, we validated NBS1 immunohistochemical (IHC) results by real-time RT-PCR. The clinical significance of increased NBS1 expression in OSCC and NO-HNSCC was analyzed and compared, and the correlation between the phosphorylation level of Akt and NBS1 expression was examined in representative OSCC samples. Our results confirm the reliability of NBS1 IHC as a biomarker in OSCC, illustrate a comprehensive profile of NBS1 in OSCC and NO-HNSCC, and establish the prognostic role of NBS1/Akt axis in different primary sites of head and neck cancers.

Materials and Methods

Study population. A total of 125 patients with OSCC who underwent surgical treatment at Taipei Mackay Memorial Hospital and Taipei Veterans General Hospital between January 2001 and December 2004 were enrolled in this study. To compare the significance of NBS1 overexpression in OSCC and NO-HNSCC, the published data from 81 patients diagnosed as locally advanced squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and larynx (T3-4NxM0) in Taipei Veterans General Hospital were also included in analysis.[35] Informed consent was obtained prior to patient enrollment, and this was approved by the Institutional Review Board of Taipei Veterans General Hospital. For OSCC, patients with T1-2N0M0 disease received wide excision of primary tumor (n = 40), and those with T3-4NxM0 disease received primary tumor excision, neck dissection, and post-operative radiotherapy (PORT) (n = 85), or primary chemoradiotherapy (CRT) (n = 23). For NO-HNSCC, all cases were advanced disease and decided to receive primary CRT (n = 58). Primary CRT included cisplatin-based chemotherapy and radiotherapy for primary tumor and neck up to 70 Gy. PORT included at least 60 Gy for the primary site, and 50 Gy for neck nodes. IHC analysis was performed in all 206 OSCC cases, and conventional RT-PCR and real-time RT-PCR was performed in 65 cases with frozen samples available for RNA extraction for validating the IHC results. Western blot analysis was performed in five representative cases with NBS1 overexpression, that is NBS1 IHC ++ and real-time PCR >2-fold increase in mRNA expression. Thus, among the 206 cases, all were analyzed by IHC; 65 by conventional RT-PCR, real-time RT-PCR, and IHC; and five by IHC, conventional RT-PCR, real-time RT-PCR, and Western blotting. The clinical characteristics of 206 HNSCC, including 148 OSCC and 58 advanced NO-HNSCC patients are illustrated in Table 1.

RNA purification, cDNA synthesis, semi-quantitative PCR, and quantitative real-time PCR analysis. Fresh frozen samples of

Table 1. Clinical characteristics and univariate survival analysis of OSCC, advanced NO-HNSCC, and HNSCC patients

Variables	OSCC (n = 148)			Advanced NO-HNSCC (n = 58)			Overall HNSCC (n = 206)		
	Patient no. (% of OSCC)	Median OS (months)	P-values*	Patient no. (% of NO-HNSCC)	Median OS (months)	P-values*	Patient no. (% of HNSCC)	Median OS (months)	P-values*
Age (years)									
≤50	58 (39.2)	—†	0.961	37 (63.8)	15.4	0.021	95 (46.1)	20.4	0.133
>50	90 (60.8)	—		21 (36.2)	26.0		111 (53.9)	27.0	
Gender									
Male	138 (93.2)	—	0.607	55 (94.8)	20.4	0.656	193 (93.7)	26.0	0.737
Female	10 (6.8)	—		3 (5.2)	—		13 (6.3)	—	
Site									
OSCC	148 (100)	—	0.230	0	NA	0.387	148 (71.8)	—	0.131
Buccal mucosa	64 (43.2)	20.4		0	NA		64 (31.1)	20.4	
Gingival	23 (15.5)	—		0	NA		23 (11.2)	—	
Tongue	52 (35.2)	—		0	NA		52 (25.2)	—	
Others OSCC‡	9 (6.1)	—		0	NA		9 (4.3)	—	
NO-HNSCC	0	NA		58 (100)	20.4		58 (28.2)	20.4	
Oropharynx	0	NA		11 (19.0)	26.0		11 (5.3)	26.0	
Hypopharynx	0	NA		28 (48.3)	20.4		28 (13.6)	20.4	
Larynx	0	NA		19 (32.7)	22.4		19 (9.3)	22.4	
T stage									
T1-2	54 (36.5)	—	0.090	0		NA	54 (26.2)	—	0.044
T3-4	94 (63.5)	—		58 (100)			152 (73.8)	22.4	
N stage									
N0	91 (61.5)	—	<0.001	15 (25.9)	26.0	0.067	106 (51.5)	—	<0.001
N1-3	57 (38.5)	18.4		43 (74.1)	20.4		100 (48.5)	18.4	
Treatment strategy									
Surgery	40	—	0.004	0		NA	40 (19.4)	—	0.007
Surgery + PORT	85	26.0		0			85 (41.3)	26.0	
Primary CRT	23	—		58 (100)			81 (39.3)	22.4	
NBS1 IHC result									
0 to +	90 (60.8)	—	<0.001	27 (46.5)	26.0	0.009	117 (56.8)	—	<0.001
++	58 (39.2)	14.0		31 (53.5)	15.4		89 (43.2)	14.4	

*Estimated by log-rank test; †the median overall survival was not reached; ‡including mouth floor and hard palate. CRT, chemoradiotherapy; HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry; NA, not applicable; NO-HNSCC, non-oral-cavity head and neck squamous cell carcinoma; OS, overall survival; OSCC, oral squamous cell carcinoma; PORT, post-operative radiotherapy.

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tumor and corresponding non-cancerous matched tissue (NCMT) embedded in RNAlater (Ambion, Austin, TX, USA) were available in 65 cases for RNA purification and cDNA synthesis. After being homogenized with MagNA Lyser Rotor (Roche, Basel, Switzerland), total RNA was extracted with an RNAeasy kit (Qiagen, Hilden, Germany). One μg of RNA was used to synthesize cDNA as described.[35]

Semi-quantitative PCR reactions were performed in the ThermoHybaid Cycler (ABgene, Epsom, UK). TBP (TATA box binding protein) was selected as a control for RT-PCR experiments.[35] In order to further confirm the levels of NBS1 mRNA in OSCC samples, quantitative real-time RT-PCR was performed in the ABI PRISM7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the preset PCR program. The target quantity was measured by identifying the threshold cycle number (C_T). TBP was selected as an internal control for real-time PCR experiments. Transcript levels were normalized to those of TBP to account for variability in the amount of cDNA in each sample, and relative expression levels were calculated using the $2^{-\Delta\Delta C}{}_T$ method.[37] The primer sequences used in the experiments are shown in Table 2.

Tissue microarray construction. A high-density tissue microarray (TMA) was constructed using formalin-fixed, paraffin-embedded specimens of OSCC and NCMT as previously described.[38] Two specialists evaluated the tissue sections by routine H&E staining and identified the regions of interest. The regions of interest were sampled by removing a 3-mm tissue core and then implanted into a recipient paraffin block to create an array containing a total of 40 tissue cores that included 20 cores of OSCC and 20 cores of NCMT on one slide. The constructed TMA was used for subsequent immnunohistochemical analysis.

Table 2. Primers for RT-PCR and real-time PCR analysis

		Sequence (5′ → 3′)	Amplicon (bp)
RT-PCR			
NBS1	F	GAAATTGAGTTCCGCAGTTGTC	572
	R	GGATTCTCATCTTAGCCAAAG	
TBP	F	CACGAACCACGGCACTGAT	398
	R	CGTCGTCTTCCTGAATCCCT	
Real-time PCR			
NBS1	F	ATGGAGGCCATATTTCCATGAC	152
	R	CAAGCAGCCAGAACTTGGAAG	
TBP	F	TGGTGGTGTTGTGAGAAGATGG	76
	R	CGGTGGGCACTTACAGAAGG	

TBP, TATA box binding protein.

Immunohistochemistry (IHC) and scoring. After deparaffinization of TMA slides, the IHC procedure was performed as described with an anti-NBS1 antibody (NB100-143; Novus Biologicals, Littleton, CO, USA) or an IHC-specific anti-phosphorylated Akt (ser 473) antibody (#9277S; Cell Signaling Technology, Beverly, MA, USA).[35] The IHC staining was independently scored by two experienced specialists who were blinded to the clinical data. If there was discordance with IHC scoring, a pathologic peer review was performed to consolidate the result into a final score.

The NBS1 IHC scoring was modified from our recent report.[35] We previously categorized NBS1 expression from 0 to +++. Because there was no difference in clinical presentation between + and ++,[35] we therefore grouped the original score + and ++ into +, and changed the original +++ to ++ to simplify the NBS1 scoring system in this study from 0 to ++. The representative results of NBS1 IHC are illustrated in Figure 1: 0, no appreciable staining in cells (Fig. 1a); +, appreciable nucleus staining without cytoplasmic staining or with cytoplasmic staining in less than 25% of cells (Fig. 1b); and ++, significant nucleus staining and more than 25% of cells with cytoplasmic staining (Fig. 1c). The IHC results of phosphorylated Akt were interpreted as previously defined.[35]

Protein extraction and Western blot analysis. For extraction of proteins from OSCC and NCMT, 500 mg of samples were lysed and were clarified by centrifugation. The protein content was determined by Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). For Western blot analysis, 50 μg of protein extracts 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), phospho-Akt (Ser 473) antibody (#9271S; Cell Signaling Technology), Akt antibody (#9272; Cell Signaling Technology), phospho-mammalian target of rapamycin (mTOR) (Ser 2448) antibody (#2971; Cell Signaling Technology), and an anti-γ-tubulin antibody (sc-7396; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as loading control. Signals were developed using an ECL chemiluminescence kit (Amersham Biosciences, Little Chalfont, UK). Data shown here are representative of two or more experiments.

Statistical analysis. The Spearman correlation coefficient test was used to determine the degree of concordance of different NBS1 expression assays, with a value of >0.6 deemed to be a significant correlation. The independent Student's *t*-test or ANOVA was used to compare relative NBS1 mRNA expression between groups. The Kaplan–Meier estimate was used for survival analysis, and the log-rank test was selected to compare the

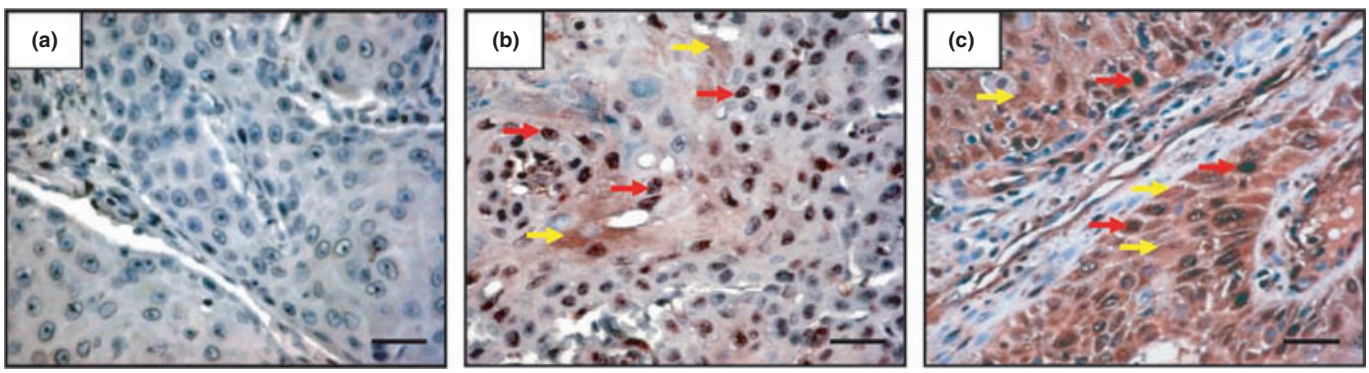

Fig. 1. Immunohistochemical analysis of NBS1 protein expression in oral squamous cell carcinoma (OSCC) samples. (a) No NBS1 protein expression (immunohistochemical [IHC] level 0); (b) nucleus plus weak cytoplasmic staining (IHC level +); (c) nucleus plus strong cytoplasmic staining (IHC level ++). Red arrows indicate nucleus staining, whereas yellow arrows indicate cytoplasmic staining. The scale bars represent 100 μm in each panel.

cumulative survival durations in different patient groups. The Cox's proportional hazards model was applied in multivariate survival analysis to test the independent prognostic factors. The χ^2-test (for values >5) or Fisher's exact test (for values ≤5) was applied for comparison of dichotomous variables. The level of statistical significance was set at 0.05 for all tests.

Results

Increased NBS1 mRNA expression in OSCC patients. To investigate the NBS1 expression profile in OSCC, semi-quantitative RT-PCR analysis was performed to screen the mRNA level of NBS1 in the available 65 pairs of OSCC and NCMT samples. A significantly increased NBS1 expression was observed in 26 (40%) OSCC cases (a representative picture is shown in Fig. 2a). Quantitative real-time RT-PCR analysis was applied for confirmation and the result showed that increased NBS1 mRNA expression (>2-fold) was observed in the NBS1 RT-PCR-positive cases (26 cases, 40%); whereas there was no or less than two-fold increase in the samples of NBS1 RT-PCR-negative cases (Fig. 2b). This result indicates that the increased NBS1 mRNA expression (>2-fold) could be identified in a significant proportion (40%) of OSCC patient samples.

Excellent correlation between NBS1 mRNA expression and IHC scoring/protein expression in OSCC samples. We previously demonstrated that increased NBS1 expression was observed in a significant percentage (~45%) of advanced HNSCC cases.[35] To validate the IHC results of NBS1 expression, IHC analysis of NBS1 expression in tumor tissues and NCMT of 125 surgically treated OSCC patients (including the 65 pairs of OSCC and NCMT samples whose mRNA were analyzed) was performed and the correlation between NBS1 mRNA expression and IHC scores was analyzed. Among these 125 OSCC cases, 35 samples (28%) showed IHC level 0 (Fig. 1a), 38 cases (30.4%) were IHC level + (Fig. 1b), whereas 52 samples (41.6%) showed NBS1 IHC ++ (Fig. 1c; defined as increased NBS1 expression). A significantly increased NBS1 mRNA level measured by real-time RT-PCR was observed in the NBS1 IHC ++ group (mean value of NBS1 $2^{-\Delta\Delta CT}$ of IHC ++ *vs* 0 to + was 11.55 ± 7.62 *vs* 0.77 ± 0.59, $P < 0.001$), and an excellent correlation between NBS1 mRNA expression levels and IHC scores was shown (mean values of NBS1 $2^{-\Delta\Delta CT}$ of IHC ++, +, and 0 were 11.55 ± 7.62, 1.17 ± 0.38, and 0.18 ± 0.13, respectively; $P < 0.001$, Fig. 3a). The degree of concordance between real-time RT-PCR and IHC scores was high (Spearman correlation coefficient $\rho = 0.936$). All NBS1 IHC ++ tumor samples demonstrated a greater than 2-fold increase in NBS1 mRNA expression (Fig. 3a). Western blot analysis of five representative pairs of OSCC samples with NBS1 overexpression (IHC ++/>2-fold increase in mRNA) also showed the increased NBS1 protein levels in tumor tissues (Fig. 3b). The excellent correlation between the NBS1 mRNA and IHC scoring/Western blot result confirmed the robustness of IHC results. These results also indicate the clinical application of NBS1 IHC scores as a biomarker of OSCC.

Clinical significance of increased NBS1 expression in OSCC, NOHNSCC, and overall HNSCC patients. To compare the prognostic

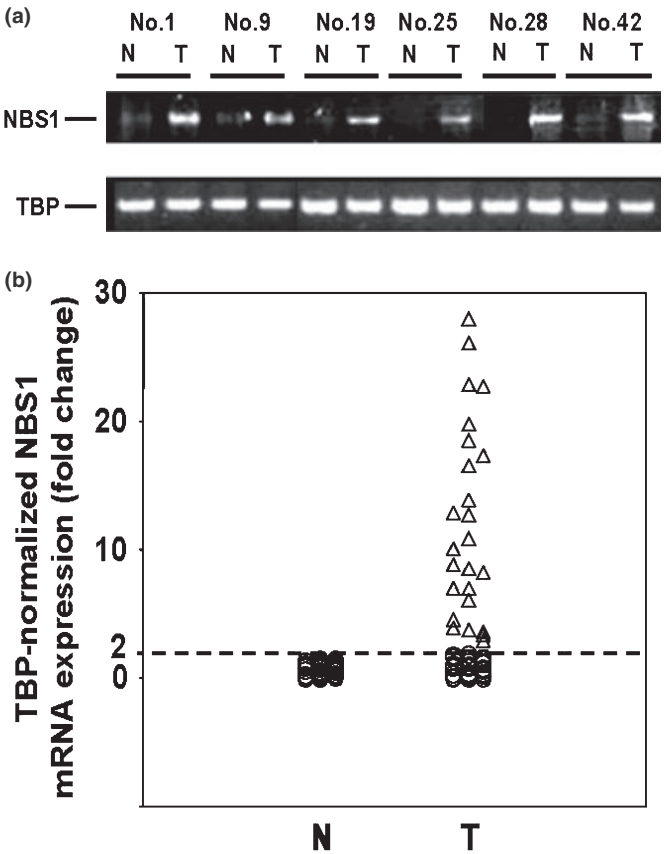

Fig. 2. Increased NBS1 mRNA expression in oral squamous cell carcinoma (OSCC) patient samples. (a) NBS1 mRNA expression of six representative pairs of OSCC cases was analyzed by RT-PCR. TBP (TATA box binding protein) was selected as a loading control. (b) Relative NBS1 mRNA expression levels of tumor (T) and non-cancerous matched tissues (N) estimated by real-time RT-PCR analysis of 65 OSCC cases. TBP was used as an internal control. The triangles (Δ) indicate the cases with NBS1 RT-PCR-positive results.

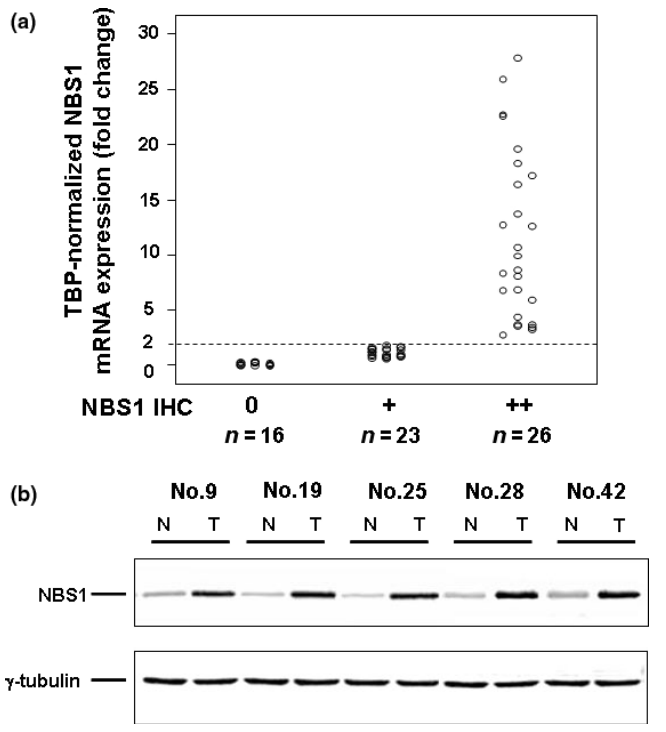

Fig. 3. Correlation between NBS1 mRNA expression and IHC scoring/protein expression in oral squamous cell carcinoma (OSCC) cases. (a) Relative NBS1 mRNA expression levels of OSCC samples estimated by real-time PCR analysis of 65 OSCC cases with NBS1 immunohistochemical (IHC) scores from 0 to ++. TBP (TATA box binding protein) was used as an internal control. (b) Western blot analysis of NBS1 protein levels in five representative pairs of OSCC cases (NBS1 IHC ++/>2-fold increase in mRNA expression). γ-Tubulin was used as a control for protein loading.

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significance of increased NBS1 expression in OSCC and HNSCC from different primary sites, we incorporated the published data of 81 advanced HNSCC for analysis.[35] Therefore, Kaplan–Meier survival analysis was performed in 148 OSCC, 58 advanced NO-HNSCC, and 206 HNSCC cases. In OSCC, patients with NBS1 IHC ++ were associated with a significantly shorter overall survival period compared with the IHC 0 and +. The 2-year overall survival rate was 30.0%, 72.8% and 78.2% in NBS IHC level ++, +, and 0 patients, respectively (Fig. 4a). We therefore categorized patients into two groups: NBS1 IHC level ++ *versus* 0 to +. Survival analysis demonstrated that the NBS1 IHC ++ patients had a significantly worse outcome ($P < 0.001$, Fig. 4b). A similar trend was observed in 58 advanced NO-HNSCC cases (Fig. 4c,d). Furthermore, the prognostic effect of NBS1 overexpression was also demonstrated in 206 HNSCC cases (Fig. 4e,f). Univariate survival analysis was also performed for other clinical variables, and the results showed that in addition to NBS1 overexpression, neck node involvement and treatment strategy were significant prognostic factors for 148 OSCC cases ($P < 0.001$, 0.004, respectively); a younger age predicted poor prognosis in 58 NO-HNSCC cases ($P = 0.021$); and advanced T stage, neck node metastasis, and treatment strategy were prognostic factors in 206 HNSCC cases ($P = 0.044$, <0.001, 0.007, respectively) (Table 1). To evaluate the independent prognostic effect, multivariate survival analysis by Cox proportional hazard model was carried out. The results showed

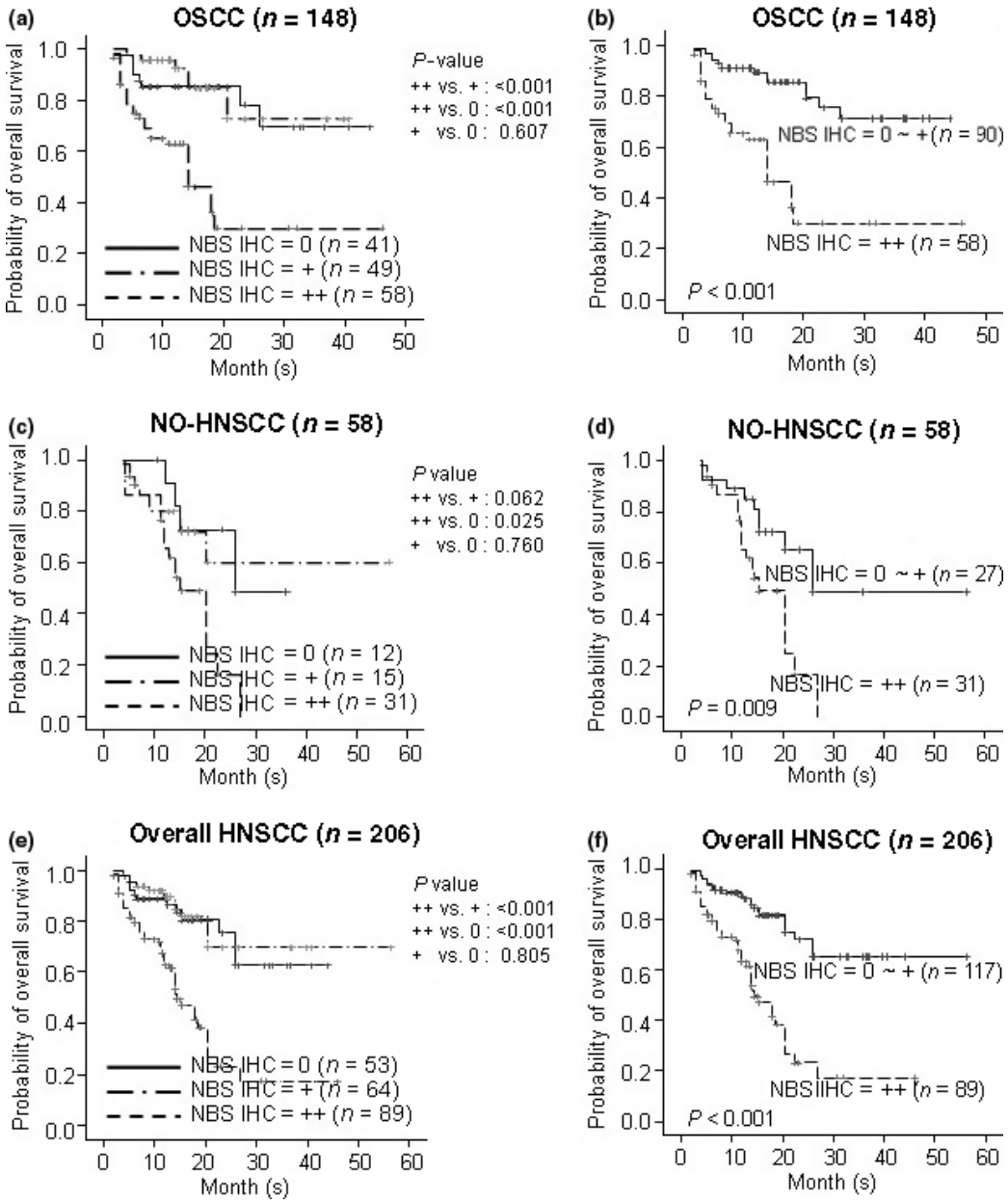

Fig. 4. Prognostic significance of increased NBS1 expression in 148 oral squamous cell carcinoma (OSCC), 58 non-oral-cavity head and neck squamous cell carcinoma (HNSCC) (NO-HNSCC), and 206 HNSCC patients. (a,b) Statistical analysis for OSCC cases. (a) Individual comparison of prognostic value between NBS1 immunohistochemical (IHC) level ++ *vs* + *vs* 0. The *P*-value between each comparison is shown in the inset. (b) Statistical comparison between NBS1 IHC level ++ *vs* 0 to + grouped together. (c,d) Analysis for NO-HNSCC cases. (c) Comparison of overall survival between NBS1 IHC level ++ *vs* + *vs* 0. (d) Comparison between NBS1 IHC level ++ *vs* level 0 to +. (e,f) Analysis for overall HNSCC cases. (e) Comparison between NBS1 IHC level ++ *vs* + *vs* 0. (f) Comparison between NBS1 IHC level ++ *vs* 0 to +.

that N status and increased NBS1 expression were independent markers for predicting overall survival in OSCC ($P = 0.042$, <0.001, respectively); a younger age and NBS1 overexpression were independent prognostic factors in NO-HNSCC ($P = 0.041$, 0.020, respectively); whereas neck node involvement and NBS1 overexpression predicted overall patients' outcome independently ($P = 0.008$, <0.001, respectively) (Table 3). The correlation between NBS1 overexpression and clinical factors were also investigated in different patient groups. In OSCC, increased NBS1 expression was associated with advanced T stage and recurrent/metastasis ($P < 0.001$, <0.001, respectively). Recurrence/metastasis was related to NBS1 overexpression in NO-HNSCC ($P < 0.001$), whereas advanced T stage, lymph node involvement, and recurrence/metastasis correlated with NBS1 expression in overall HNSCC ($P < 0.001$, 0.028, and <0.001, respectively) (Table 4). Collectively, these results delineate the clinical significance of NBS1 overexpression in OSCC or NO-HNSCC irrespective of the primary tumor location.

To further confirm the prognostic effect of NBS1-overexpressed OSCC, subgroup analysis was performed. First, we stratified the according to T stage or N status. The results showed that increased NBS1 expression was a prognostic marker regardless of T stage or lymph node status (Fig. 5). Second, we stratified the patients by treatment modality. The prognostic effect NBS1 overexpression was shown in patients receiving surgery, surgery + PORT, and primary CRT (Fig. 6). These results further strengthen the unique and independent role of NBS1 overexpression in predicting prognosis of OSCC cases. In summary, all the statistical analyses suggest that increased NBS1 overexpression is a useful biomarker both for OSCC and NO-HNSCC. It

Table 3. Multivariate survival analysis of OSCC, advanced NO-HNSCC, and HNSCC patients

Variables	OSCC (n = 148)		Advanced NO-HNSCC (n = 58)		Overall HNSCC (n = 206)	
	Hazard radio (95% CI)	*P*-values	Hazard radio (95% CI)	*P*-values	Hazard radio (95% CI)	*P*-values
N stage (N1-3 *vs* N-)	2.061 (1.028–4.133)	0.042	NA	NA	2.614 (1.226–3.818)	0.008
Age (<50 years *vs* ≥50)	NA	NA	2.506 (1.039–6.060)	0.041	NA	NA
Treatment strategy (surgery + PORT or primary CRT *vs* surgery)	1.675 (0.586–4.794)	0.336	NA	NA	1.647 (0.599–4.526)	0.333
NBS1 overexpression (yes *vs* no)	3.751 (1.948–7.225)	<0.001	2.673 (1.171–6.099)	0.020	3.373 (2.207–5.613)	<0.001

CI, confidence interval; CRT, chemoradiotherapy; HNSCC, head and neck squamous cell carcinoma; NA, not applicable; NO-HNSCC, non-oral-cavity head and neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma; PORT, post-operative radiotherapy.

Table 4. Correlation between NBS IHC result and clinical variables in OSCC, advanced NO-HNSCC, and HNSCC cases

Variables	OSCC (n = 148)			Advanced NO-HNSCC (n = 58)			Overall HNSCC (n = 206)		
	Patient no.	NBS1 overexpression (IHC ++)		Patient no.	NBS1 overexpression (IHC ++)		Patient no.	NBS1 overexpression (IHC ++)	
		Case no. (%)	*P*-values*		Case no. (%)	*P*-values*		Case no. (%)	*P*-values*
Age (years)									
≤50	58	20 (34.5)	0.346	37	23 (62.2)	0.077	95	43 (45.3)	0.581
>50	90	38 (42.2)		21	8 (38.1)		111	46 (41.4)	
Gender									
Male	138	56 (40.6)	0.198	55	29 (52.7)	1.000	193	85 (44.0)	0.350
Female	10	2 (20.0)		3	2 (66.7)		13	4 (30.8)	
Site									
OSCC	148	58 (39.2)	0.290	0	NA	0.206	148	58 (39.2)	0.063
Buccal mucosa	64	23 (35.9)		0	NA		64	23 (35.9)	
Gingival	23	13 (56.5)		0	NA		23	13 (56.5)	
Tongue	52	18 (34.6)		0	NA		52	18 (34.6)	
Other OSCC†	9	4 (44.4)		0	NA		9	4 (44.4)	
NO-HNSCC	0	NA		58	31 (53.5)		58	31 (53.4)	
Oropharynx	0	NA		11	7 (63.6)		11	7 (63.6)	
Hypopharynx	0	NA		28	17 (60.7)		28	17 (60.7)	
Larynx	0	NA		19	7 (36.8)		19	7 (36.8)	
T stage									
T1-2	54	9 (16.7)	<0.001	0	0 (0)	NA	54	9 (16.7)	<0.001
T3-4	94	49 (52.1)		58	31 (53.4)		152	80 (52.6)	
N stage									
N0	91	31 (34.1)	0.107	15	7 (46.7)	0.541	106	38 (35.8)	0.028
N1-3	57	27 (47.4)		43	24 (55.8)		100	51 (51.0)	
Recurrence/metastasis									
No	99	26 (26.3)	<0.001	23	7 (30.4)	0.004	122	33 (27.0)	<0.001
Yes	49	32 (65.3)		35	24 (68.6)		84	56 (66.7)	

*Estimated by Pearson χ^2-test ($n > 5$) or Fisher's exact test ($n \leq 5$); †including mouth floor and hard palate. CRT, chemoradiotherapy; HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry; NO-HNSCC, non-oral-cavity head and neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma; PORT, post-operative radiotherapy.

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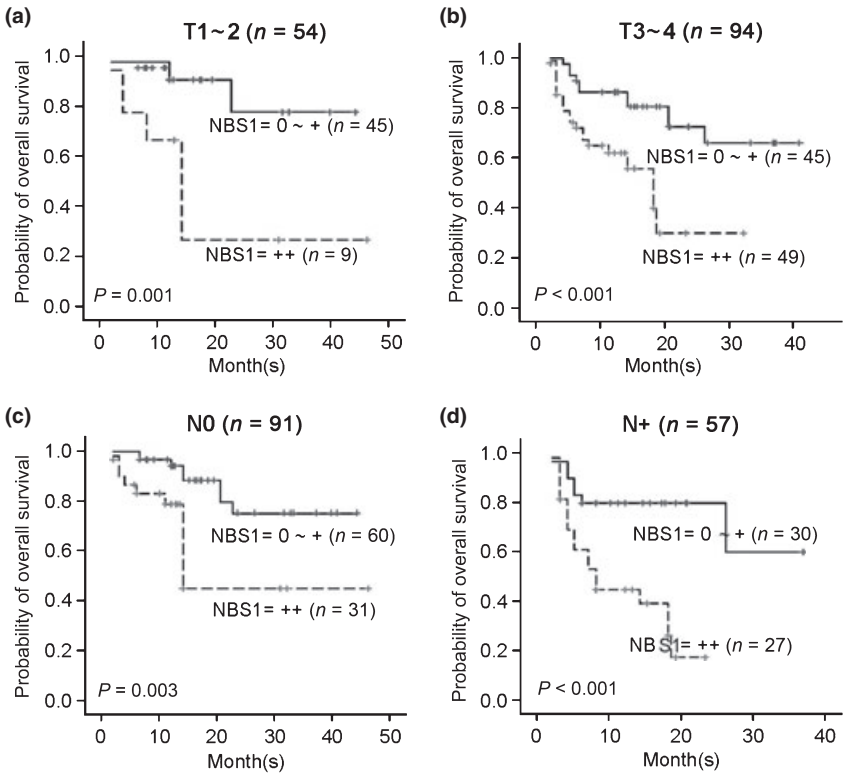

Fig. 5. Prognostic significance of NBS1 overexpression in 148 oral squamous cell carcinoma (OSCC) cases stratified by T stage and lymph node status. (a,b) Comparison of overall survival periods between NBS immunohistochemical (IHC) level ++ *vs* 0 to + in T1–2 (a) and T3–4 (b) cases. (c,d) Overall survival analysis of NBS1 expression stratified by nodal status (c, N0, no lymph node involvement; d, N+, lymph node metastasis at diagnosis).

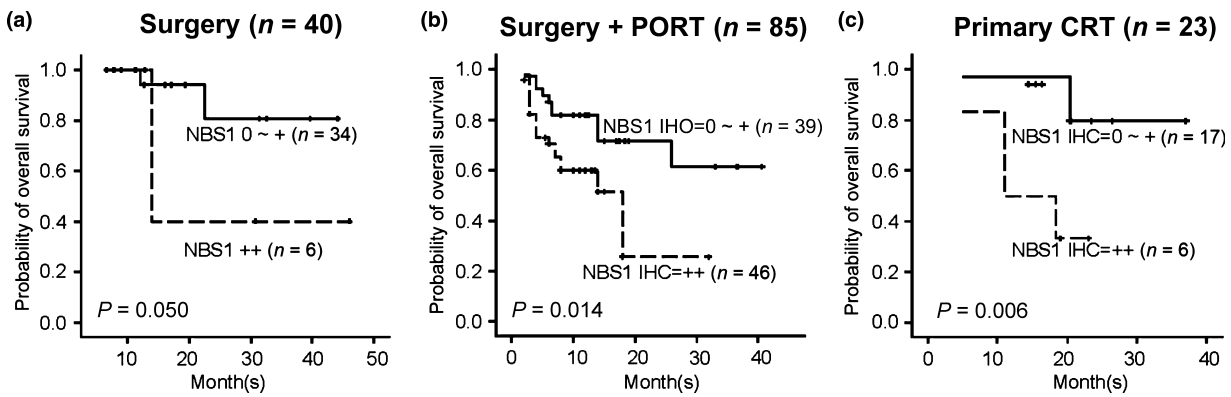

Fig. 6. Prognostic significance of NBS1 overexpression in 148 oral squamous cell carcinoma (OSCC) cases stratified by treatment modality. Comparison of overall survival periods between NBS immunohistochemical (IHC) level ++ *vs* 0 to + in patients receiving surgery (a), surgery plus post-operative radiotherapy (PORT) (b), and primary chemoradiotherapy (CRT) (c).

predicted the patients' outcome and was associated with advanced or recurrent disease.

NBS1 overexpression activated the Akt pathway in OSCC samples. We previously demonstrated that overexpression of NBS1 contributes to transformation through the activation of PI3-kinase/Akt.[33–35] In order to confirm that activation of the Akt pathway by NBS1 indeed occurs in OSCC patient samples, IHC of phosphorylated Akt was performed in 125 surgically treated OSCC cases. Predominantly cytoplasmic staining of phosphorylated Akt (right panel of Fig. 7a) was detected in 48 (92.3%) NBS1 IHC level ++ patient samples. On the contrary, negative staining of phosphorylated Akt (left panel of Fig. 7a) was shown in all 73 (100%) NBS1 IHC level 0 to + patient samples (data not shown). Western blot analysis showed the activation of Akt and its downstream target mTOR in representative OSCC patient samples (Fig. 7b). These results demonstrated the activation of Akt by NBS1 in OSCC patient samples.

Discussion

Although the prognostic significance of NBS1 overexpression identified by IHC in advanced HNSCC has been highlighted in our previous study, reports investigating NBS1 expression in cancer by IHC are relatively limited with the exception of Ehlers and Harbour who showed the prognostic role of NBS1 in uveal melanoma.[39] Therefore, validation of the NBS1 IHC results for clinical application is mandatory. Real-time RT-PCR is considered a robust and sensitive tool for routine diagnostics, and its result is usually consistent with other standard methodologies.[40] It has been applied to validate the results of IHC in tumor samples.[41] In this report, we demonstrated an excellent correlation between NBS1 mRNA expression evaluated by real-time RT-PCR and IHC scores in OSCC samples, and NBS1 IHC level ++/>2-fold increase in mRNA expression was an important risk category of OSCC. This highly consistent result not

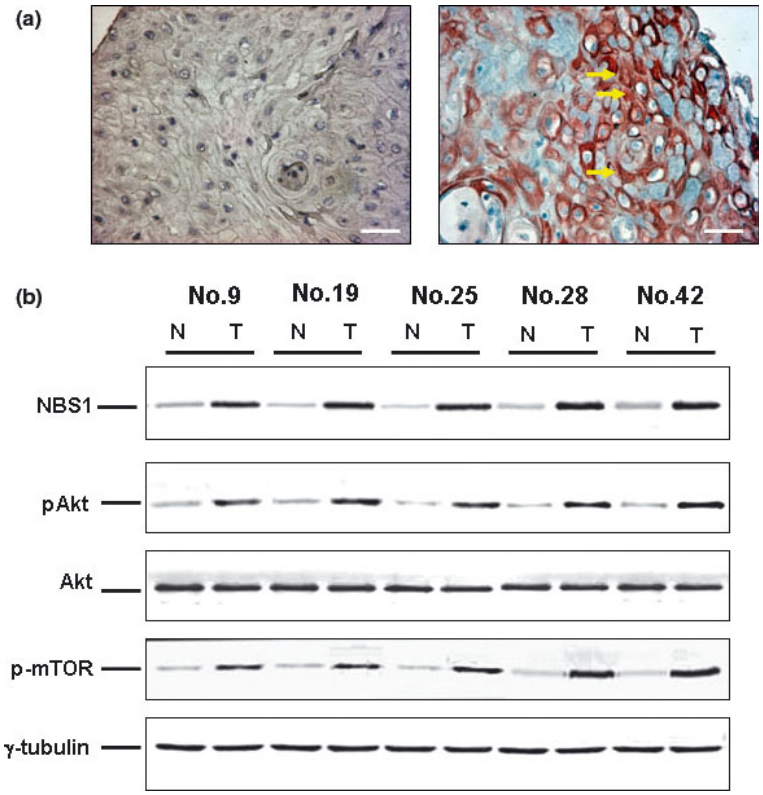

Fig. 7. NBS1 overexpression activated the Akt pathway in oral squamous cell carcinoma (OSCC) patient samples. (a) Two representative OSCC cases stained with phosphorylated Akt (pAkt). Left panel, negative pAkt staining; right panel, positive pAkt staining. The yellow arrows indicated cytoplasmic staining. The scale bars represent 100 μm in each panel. (b) Western blot analysis of NBS1, phospho-Akt, Akt, phospho-mammalian target of rapamycin (mTOR), and γ-tubulin in five representative cases with NBS1 immunohistochemical (IHC) level ++/>2-fold increase in mRNA expression. Total Akt and γ-tubulin were used as protein loading controls.

only confirms the reliability and robustness of our NBS1 IHC procedures and interpretations, but also indicates the prognostic importance and clinical practicability of NBS1 IHC in OSCC.

Because of the discrepancy in the tumor behavior and clinical outcome between OSCC and HNSCC originating from the other sites, confirmation of the biomarker proven in HNSCC is necessary before application in OSCC. In this report, increased NBS1 expression occurred in 39.2% and 53.5% of OSCC and advanced NO-HNSCC patients, respectively. The higher incidence of NBS1 overexpression in NO-HNSCC is attributed to the advanced stage of NO-HNSCC cases (T3-4NxM0). Multivariate analysis showed that NBS1 overexpression is an independent factor for predicting prognosis both in OSCC and NO-HNSCC. NBS1 overexpression is associated with an aggressive behavior (e.g. recurrence/metastasis) irrespective of primary sites. Subgroup analysis results further strengthens the critical role of NBS1 overexpression in OSCC: NBS1 is prognostically significant regardless of treatment modality, T stage, and lymph node metastasis. Collectively, our reports not only certify the role of NBS1 overexpression in OSCC, the importance of NBS1 in HNSCC from different primary sites is also displayed. Therefore, incorporation of intensive therapy (e.g. dose-intensified chemoradiotherapy) in NBS1-overexpression cases may be mandatory due to the aggressiveness.

Increased phosphorylation levels of Akt and its downstream targets have been identified in different types of human cancer,[42–44] and the PI3-kinase/Akt pathway is regarded as one of the most important oncogenic pathways in human cancers.[45] The prognostic value of activated Akt expression in OSCC has been reported.[46] We previously demonstrated that NBS1 overexpression activates PI3-kinase, which induces transformation through increasing cell proliferation and promoting cell growth by phosphorylating its various downstream target proteins (e.g. mTOR).[33,34] Increased Akt phosphorylation levels could be identified in most HNSCC patients with increased NBS1 expression, and cytoplasmic co-localization of NBS1 and phospho-Akt expression could be demonstrated in NBS1-IHC-positive tumor tissues.[35] In this report, increased Akt phosphorylation levels was identified in more than 90% of NBS1-IHC-positive OSCC patients. Activation of Akt and its downstream target mTOR was also shown in representative OSCC samples. Our report indicates that NBS1 overexpression is one of the major pathways contributing to the activation of Akt in OSCC, suggesting the critical role of the NBS1/Akt axis in OSCC progression.

In conclusion, our results demonstrate a comprehensive profile of NBS1 expression in HNSCC from different sites including OSCC and NO-HNSCC. Overexpression of NBS1 is a useful biomarker of OSCC in clinical practice using real-time RT-PCR or IHC methods, and reliability was confirmed by the near-perfect degree of concordance between these two assays. The clinical significance and prognostic role of NBS1 overexpression was shown in both in OSCC and NO-HNSCC. NBS1 is the major activator of the Akt pathway in OSCC. This discovery provides valuable information for the identification and characterization of a novel molecular marker using simple and reliable methods that can be applied in the diagnosis, prognosis, and management of OSCC patients.

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Disclosure Statement

The authors have no conflict of interest.

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