

ORIGINAL ARTICLE

Clinicopathological values of NBS1 and DNA damage response genes in epithelial ovarian cancers

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Epithelial ovarian cancers (EOCs) are highly lethal gynecological malignancies with a high recurrence rate. Therefore, developing prognostic markers for recurrence after chemotherapy is crucial for the treatment of ovarian cancers. As ovarian cancers frequently respond to DNA-damaging agents, we assessed the clinicopathological significance of key double-strand DNA break (DSB) repair genes, including *BRCA1*, *BRCA2*, *BARD1*, *ATM*, *RAD51* and *NBS1* in EOC cell lines and paraffin-embedded tissue sections from 140 EOC patients treated with cytoreductive surgery, followed by platinum-based chemotherapy. These samples were analyzed for the clinicopathological impact of DSB genes by western blot analysis, immunohistochemistry and quantitative real-time PCR. Of the DSB repair genes, *BRCA1*, *ATM* and *NBS1*, which are involved in the homologous recombination-mediated repair pathway, were related to aggressive parameters in EOC. When survival analysis was performed, *NBS1* expression exhibited an association with EOC recurrence. Specifically, increased *NBS1* expression was found in 107 out of 140 cases (76.0%) and correlated with advanced stage ($P=0.001$), high grade ($P=0.001$) and serous histology ($P=0.008$). The median recurrence-free survival in patients with positive and negative expression of *NBS1* was 30 and 78 months, respectively ($P=0.0068$). In multivariate analysis, *NBS1* was an independent prognostic factor for the recurrence of EOC. Together, these results suggest that *NBS1* is a marker of poor prognosis for the recurrence of EOC and is associated with aggressive clinicopathological parameters.

Experimental & Molecular Medicine (2015) 47, e195; doi:10.1038/emm.2015.85; published online 20 November 2015

INTRODUCTION

Epithelial ovarian cancer (EOC) is the fifth most common cause of cancer mortality in women and the leading cause of mortality among gynecological cancers.¹ Although cytoreductive surgery followed by adjuvant chemotherapy enhances clinical responses in the majority of ovarian cancer patients, many of them experience recurrence of the disease and eventually die from resistance to chemotherapy, especially to platinum-based chemotherapy.² Mechanisms that underlie platinum resistance are poorly understood and are most likely multifactorial in origin.^{3,4}

To date, poly ADP (adenosine diphosphate) ribose polymerase (PARP) inhibitor treatment is one of the most promising examples of personalized therapy for ovarian cancer.⁵ PARP inhibitor was originally developed during synthetic lethality screening of *BRCA2* mutant cells.⁶ Both *BRCA1* and *BRCA2* are involved in homologous recombination (HR), which repairs double-strand DNA breaks (DSB) in an error-free manner.⁷ In cells with *BRCA1*

or *BRCA2* mutations, a compensatory mechanism of DNA repair, such as base excision repair is necessary to restore DNA damage in place of HR. PARP is an enzyme involved in base excision repair, and hence *BRCA*-null cells showed hypersensitivity to PARP inhibitors that targeted base excision repair in general. Therefore, PARP inhibitors may be effective in treatment of ovarian cancers with *BRCA* mutations.

Approximately 10%~15% of EOCs are associated with inherited *BRCA1* or *BRCA2* mutations. However, dysfunction of the *BRCA*-related HR pathway may be more prevalent.⁸ This phenomenon characterized by HR deficiency is called 'BRCAness'.⁹ Therefore, these sporadic tumors with a BRCAness phenotype will behave similar to *BRCA1* or *BRCA2* mutant cancers and may show sensitivity to PARP inhibitors.¹⁰ Until now, BRCAness phenotypes, including the somatic mutation of *BRCA1* or *BRCA2* and the epigenetic hypermethylation of the *BRCA1* promoter have been reported in EOC.^{11–15} However, development of prognostic markers for EOC, other than

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Received 27 August 2015; accepted 31 August 2015

BRCA1 or BRCA2, for treatment with PARP inhibitors is needed for advanced patient-specific cancer therapy.

Here we asked whether mutations in HR pathway genes are associated with an improved response rate and survival after platinum-based chemotherapy. By assessing the mRNA and protein expression patterns of HR genes in EOC, we report that NBS1 is a marker of poor prognosis for EOC and has clinicopathological value.

MATERIALS AND METHODS

Cell culture of ovarian cancer cell lines and primary ovarian cancers

Human ovarian cancer cell lines OVCAR3, SK-OV-3, SNU8, SNU119 and SNU251 (*BRCA1* mutated) were obtained from the Korean Cell Line Bank and grown in monolayer cultures in RPMI 1640 supplemented with fetal bovine serum (10% v/v). The HCC1937 (*BRCA1* mutated) breast cancer cell line and the CAPAN1 (*BRCA2* mutated) pancreatic cell line were grown in RPMI 1640, and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10% v/v).

Primary ovarian cancer samples were collected from the Seoul National University Hospital from patients undergoing cytoreductive surgery. Tumor tissue was dissected onto 100-mm petri dishes containing serum-free RPMI medium supplemented with trypsin. After 30 min at 37 °C, cells were resuspended and maintained in RPMI 1640 supplemented with fetal bovine serum (10% v/v).

Patients and tissue samples

Tissue specimens for quantitative real-time PCR and immunohistochemistry (IHC) were obtained from 140 epithelial ovarian carcinomas by primary cytoreductive surgery, followed by platinum-based chemotherapy at Seoul National University Hospital between 1998 and 2005. All samples were taken from primary ovarian lesions in each patient. The disease stage of each sample was determined according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. Of the patients, 78 experienced a relapse and 35 died of ovarian cancer. Samples of normal ovarian tissue were obtained from the unaffected ovaries of 10 ovarian cancer patients (stage I or II), whose tumor was limited to the contralateral ovary. Formalin-fixed, paraffin-embedded ovarian carcinoma tissues were used for histologic evaluation. Gynecological pathologists reviewed all hematoxylin and eosin-stained sections. The Seoul National University Hospital Institutional Review Board approved this study (1006-089-414).

RNA preparation

Three sets of 1.0- μ m-thick tissue from paraffin block were dewaxed with xylene, which was removed using three changes of absolute ethanol. Total RNA was extracted from tissue using a High Pure Paraffin RNA Kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. For assessment of total RNA concentration and purity, a NanoDrop ND-1000 (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, USA) measured absorbance at 260 nm.

Quantitative real-time reverse-transcriptase PCR

Total RNA was reverse transcribed in a final volume of 40 μ l containing oligo dTs and 1 μ g of total RNA and with a Superscript II RNase H reverse Transcriptase Kit (Invitrogen, Waltham, MA, USA). Quantitative real-time reverse-transcriptase PCR was performed

with a sequence detector (StepOnePlus, Applied Biosystems, Foster City, CA, USA) based on the SYBR Premix Ex Taq assay (Takara, Otsu, Japan) according to the manufacturer's protocol. The following sequences were used as forward (F) and reverse (R) primers: *BRCA1* F: 5'-GAGGAGTTCTT-GACCCCAA-3', *BRCA1* R: 5'-CAGAATCAC CCGAGCAGGT-3'; *BRCA2* F: 5'-CAGC-GTGGAAATATCCTAAAT CTGA-3', *BRCA2* R: 5'-TTGGATGAACAGGAATACTTG-GAA-3'; *BARD1* F: 5'-TCTGTAATGGTGTGCTTCAAGG-3', *BARD1* R: 5'-GTGTCCAGC-ATCCAGAAAGG-3'; *RAD51* F: 5'-CCCTGCAGC CAGGACT-3', *RAD51* R: 5'-AG-GCCAACTGCGCGGAT-3'; *NBS1* F: 5'-GGTGGGAAGGACCTGATGATAG-3', *NBS1* R: 5'-GGGCG AGGCTCATCGAT-3'; and glyceraldehyde-3-phosphate dehydrogenase F: 5'-TGGTCACCAGGGCTGCTTTTA-3', glyceraldehyde-3-phosphate dehydrogenase R: 5'-TCCTGGAAGATGGTGATGGGA TTT-3'. Cycling conditions used in this PCR series were 95 °C for 10 min to activate DNA polymerase, followed by 40 cycles at 95 °C for 15 s, 55 °C for 20 s and 72 °C for 10 s.

All specimens were analyzed in duplicate and the mean values were used for quantification. The quantification value of *BRCA1*, *BRCA2*, *BARD1*, *RAD51*, *ATM* or *NBS1* mRNA was described relative to *GAPDH* mRNA. To exclude false positives, we used the mean relative mRNA value plus 2 s.d. of expressed mRNA in EOC as the cutoff value to detect expression of a corresponding gene. In this manner, values above the cutoff were defined as positive expression for the corresponding mRNA.

Western blot analysis

Protein concentrations were measured by Bradford protein assay before analysis. Total cell lysate (30–150 μ g of protein) was separated by 8% or 12% polyacrylamide SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking non-specific binding sites for 1 h with 5% non-fat milk in TBS-T (Tris-buffered saline, 0.1 % Tween 20), the membrane was incubated at 4 °C overnight with primary antibodies against *BRCA1* antibody (Oncogene, La Jolla, CA, USA), *BRCA2* (Calbiochem, La Jolla, CA, USA), *NBS1* (Santa Cruz Biotechnology, Dallas, TX, USA) and β -actin (Santa Cruz Biotechnology). The blot was washed three times with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody at room temperature for 1 h.

IHC analysis

Paraffin blocks from ovarian carcinoma were sectioned at 4 μ m adjacent to hematoxylin and eosin-stained sections. Samples were deparaffinized in xylene, rehydrated with graded ethanol and washed in distilled water. The sections were then placed in 10 mM citrate buffer (pH 6.0) and boiled in a microwave for epitope retrieval. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% H₂O₂ for 10 min. Following the blocking procedure with 20% goat serum and 1% bovine serum albumin in phosphate-buffered saline at room temperature for 1 h, sections were incubated with primary antibody against *NBS1* at a dilution of 1:100 in a humidifying chamber at 4 °C overnight. Next the sections were washed in phosphate-buffered saline for 5 min at room temperature and subsequently stained by the labeled streptavidin biotin method as suggested by the manufacturer of the Dako LSAB Kit (Dako, Agilent Technologies, Glostrup, Denmark), and were visualized using 3,3'-diaminobenzidine. The sections were then counterstained with hematoxylin. Slides were subjected to the same staining procedure without addition of primary antibodies as a negative control. Microscopy fields from each stained section were randomly sampled.

In the majority of cases, NBS1 antibody staining was nuclear. However, a few specimens showed cytoplasmic staining. The distribution of the percentage of positive staining was recorded and scored as previously reported.¹⁶ Briefly, a score of 0 indicated no appreciable staining in cells, 1 indicated appreciable nuclear staining without cytoplasmic staining or with cytoplasmic staining in <25% of cells and 2 indicated significant nuclear staining and >25% of cells with cytoplasmic staining.

Statistical analysis

The relationships between categorical variables were assessed using the χ^2 -test. The Student's *t*-test was used to compare relative mRNA expression levels between groups. Recurrence-free survival (RFS) was estimated using the Kaplan–Meier method and the differences in survival were compared using the log-rank test. Multivariate analysis was performed using the Cox regression method. A *P*-value of <0.05 was considered statistically significant. Data were analyzed using SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

BRCAness gene expression levels in EOC cells

Western blot analysis was performed to assess the expression profiles of the genes involved in the DSB repair pathway, including *BRCA1*, *BRCA2*, *BARD1*, *RAD51*, *NBS1* and *ATM* in OVCAR3, SK-OV-3, SNU8, SNU119 and SNU251 ovarian cancer cells. The HCC1937, *BRCA1*-deficient breast cancer cell line, the CAPAN1, *BRCA2*-deficient cell line, and HeLa cells, which is positive for both *BRCA1* and *BRCA2*, were used as controls. As expected, HCC1937 cells were negative for *BRCA1* and CAPAN1 cells were negative for *BRCA2*. One of the ovarian cancer cell lines investigated, SNU-251, did not express *BRCA1*. Notably, mRNA levels of *NBS1*, *BARD1*, *ATM*, *RAD51*, *BRCA1* and *BRCA2* were variable among the cell lines tested (Figure 1a).

Western blot analysis showed that, similar to the mRNA results, the SKOV3 and SNU119 cells, in addition to the SNU251 and HCC1937 cells, did not have *BRCA1* protein. Interestingly, SNU119 cells were negative for both *BRCA1* and *BRCA2*. In conclusion, mRNA and protein levels of *NBS1*, *BRCA1*, *BRCA2* and *RAD51* in ovarian cancer cell lines were variable (Figure 1b).

BRCAness gene expression levels in EOC patients

Next, we assessed the mRNA levels of *BRCA1*, *BRCA2*, *RAD51* and *NBS1* in 140 ovarian cancer patient samples. When compared with those in normal ovary tissue, all six mRNA levels were altered (Table 1 and Figure 2). The mRNA level of *NBS1* exhibited a significant difference between normal and cancer lesions in EOC patient samples (Figure 2a). In simultaneous semi-quantitative reverse-transcriptase PCR analysis, *BRCA1* and *ATM* expression levels displayed a statistically significant difference between normal and tumor samples, in addition to expression of *NBS1* (Figure 2b and Table 1). Interestingly, *BRCA1* and *NBS1* were overexpressed in tumors, whereas *ATM* expression was reduced (Table 1).

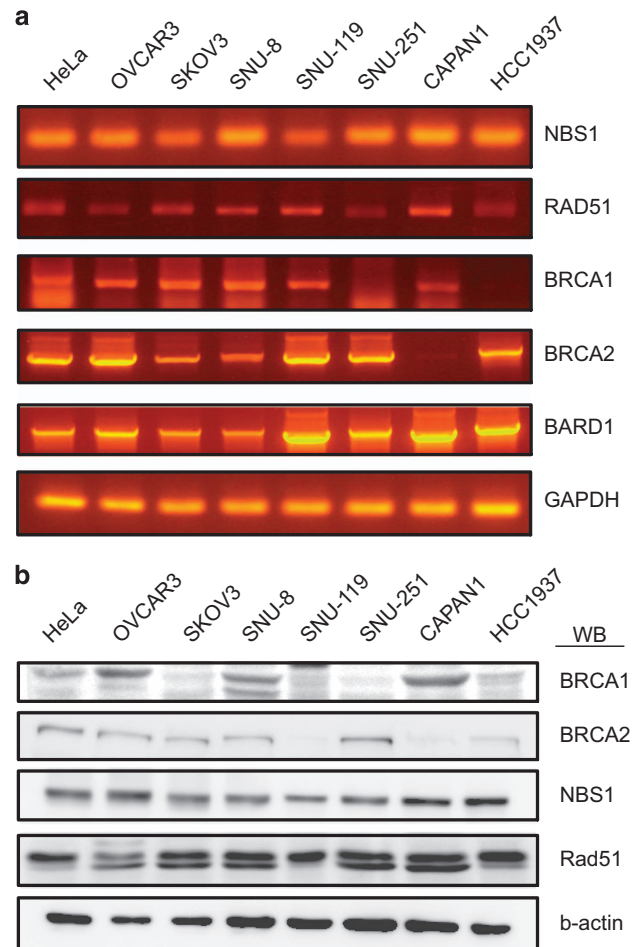


Figure 1 (a) mRNA expression profiles of *BRCA1*, *BRCA2*, *BARD1*, *ATM*, *NBS1* and *RAD51* in EOC cell lines. *BRCA1* and *BRCA2* mutated cancer cell lines HCC1937 and CAPAN1, respectively, were used for controls. *GAPDH* was used as a loading control. (b) Western blot analysis in EOC cell lines, and *BRCA1* and *BRCA2* mutated cancer cell lines. Anti- β -actin was used as a loading control.

Table 1 mRNA levels of DNA damage response genes in ovarian tissue samples

Gene	Median mRNA level (range) of normal ovary (n = 10)	Median mRNA level (range) of ovarian cancer (n = 140)
<i>BRCA1</i>	1 (0.71–2.11)	2.26 (0.08–8.25)
<i>BRCA2</i>	1 (0.57–1.62)	1.95 (0.16–10.12)
<i>BARD1</i>	1 (0.51–1.56)	0.51 (0.01–1.30)
<i>RAD51</i>	1 (0.35–3.21)	2.03 (0.60–5.87)
<i>NBS1</i>	1 (0.18–1.98)	3.32 (0.32–28.22)
<i>ATM</i>	1 (0.85–1.62)	0.76 (0.22–1.98)

Correlation between NBS1 levels and patient survival

Next, IHC staining was performed on 140 paraffin-embedded tumor samples (Table 2). The age of cancer patients ranged from 18 to 79 years (median, 51.5 years). All of the cancer patients analyzed were at stage Ic or higher, and hence

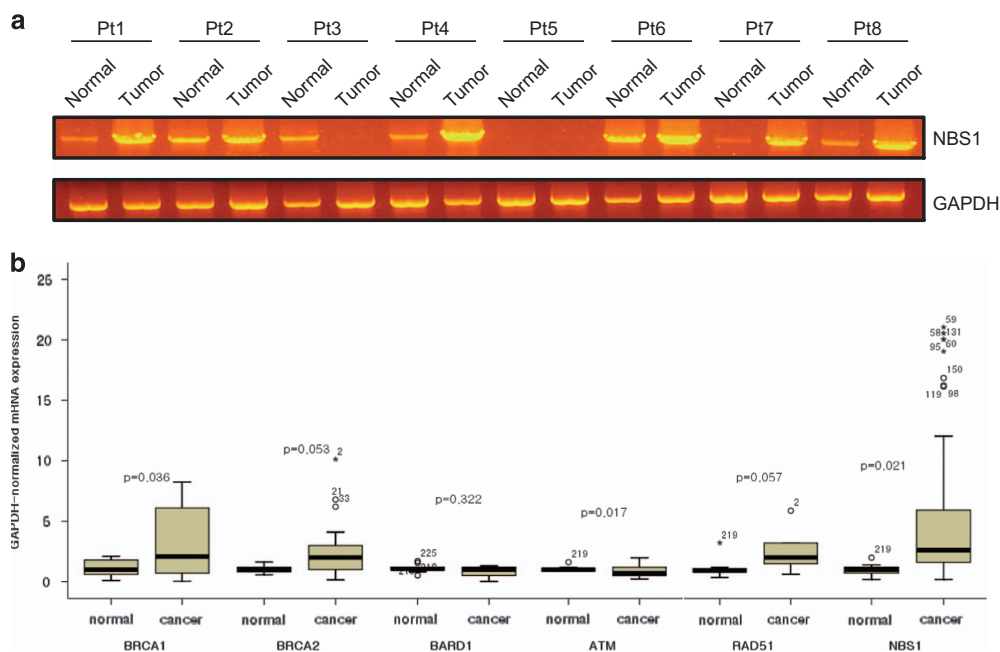


Figure 2 (a) *NBS1* mRNA expression profiles in EOC patient samples. *NBS1* mRNA expression of eight representative pairs of EOC cases was analyzed by reverse-transcriptase PCR. *GAPDH* was included for normalization. Tumor (Tumor) and non-cancerous matched tissues (Normal) were compared in all cases. (b) The mRNA expression patterns of indicated genes in 140 EOC patient samples compared with that in normal ovary (mean \pm s.e.m.). All *P*-values were obtained by Student's *t*-test between groups.

Table 2 Clinicopathological characteristics of ovarian cancer patients

	Number of patients (%)
Median age (year, range)	51.5 (18–79)
<i>FIGO</i> stage	
Ic	23 (16.4)
II	13 (9.3)
III	86 (61.4)
IV	18 (12.9)
<i>Histology</i>	
Serous	82 (58.6)
Mucinous	21 (15.0)
Endometrioid	20 (14.3)
Clear cell	15 (10.7)
Others	2 (1.4)
<i>Grade</i>	
1	23 (16.4)
2	38 (27.1)
3	79 (56.4)
<i>Residual tumor</i>	
Residual tumor \leq 1 cm	73 (52.1)
Residual tumor > 1 cm	67 (47.9)
<i>Clinical response</i>	
Complete response	91 (65.0)
Partial response	16 (11.4)
Stable disease	15 (10.7)
Progressive disease	18 (12.9)

underwent primary cytoreductive surgery, followed by taxane and platinum chemotherapy. None of the patients underwent conservative surgery. Serous cystadenocarcinoma (57.5%) was the most common histology. Cytoreductive surgery was

optimal (\leq 1 cm residual disease) in 51.9% of cases. More than two-thirds of the patients had a complete response to the first-line treatment, yet half of them relapsed.

NBS1 expression levels were associated with RFS in the EOC patients treated with cytoreductive surgery followed by platinum-based chemotherapy, whereas *BRCA1*, *BRCA2*, *BARD1*, *ATM* and *RAD51* mRNA levels were not. Relative *NBS1* mRNA expression was correlated with IHC staining score, which reflects the protein expression level (Figure 3a). An analysis of the relationship between the levels of *NBS1* and the clinicopathological parameters of 140 tumors was analyzed (Table 3) and showed that positive *NBS1* expression was associated with advanced stage ($P=0.001$), serous histology ($P=0.008$), high grade ($P=0.001$) and residual tumor ($P=0.021$).

Univariate survival analysis showed that *NBS1* immunoreactivity was a significant prognostic factor for RFS ($P<0.001$), whereas *BRCA1*, *BRCA2* and *RAD51* expression was not ($P=0.059$, $P=0.633$ and $P=0.222$, respectively). Patients immunoreactive for *NBS1* (score 2) had a median RFS of 30 months (range, 3–48 months), whereas those lacking *NBS1* expression (score 0 and 1) had a median RFS of 78 months (range, 2–115 months; Figure 3b). Multivariate survival analysis indicated that the *NBS1* level was an independent prognostic factor for predicting recurrence of ovarian cancer (Table 4). RFS curves stratified by *NBS1* mRNA expression and *NBS1* protein expression corroborated that *NBS1* has prognostic value for RFS in EOC (Figure 4a and b).

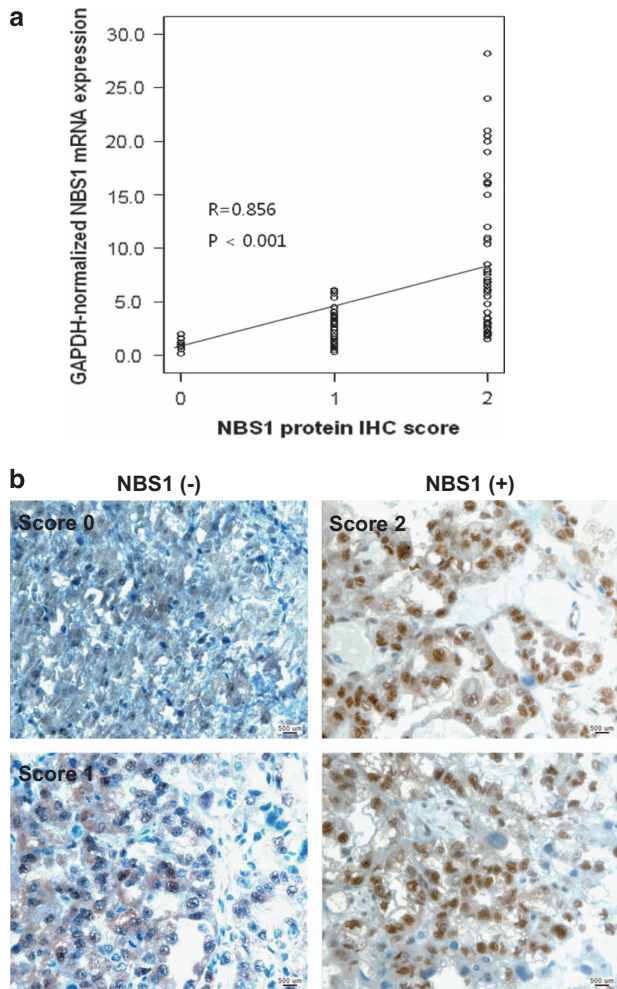


Figure 3 Correlation between *NBS1* mRNA expression and immunohistochemical (IHC) scoring of *NBS1* in primary EOC tissues. (a) Relative *NBS1* mRNA expression levels of 140 EOC patient samples estimated using real-time reverse-transcriptase PCR (RT-PCR) analysis were correlated with IHC scores 0, 1 and 2. *GAPDH* was used as an internal control for real-time RT-PCR analysis. (b) Representative positive and negative immunohistochemistry images of *NBS1* in paraffin-embedded EOC samples. Images were taken on an Olympus BX-51 microscope under $\times 100$ magnification. Scale bar, 500 μm .

DISCUSSION

In this study, we asked whether the genes involved in HR affect cancer aggressiveness and EOC prognosis in patients after platinum-based chemotherapy. We found that *NBS1* expression was heterogeneous in different EOCs and an increased *NBS1* level was a significant marker of poor prognosis in EOC patients treated with cytoreductive surgery followed by platinum-based chemotherapy.

Anti-neoplastic activity of platinum results from its DNA cross-linking activity. Normal cells cope with the DNA cross-links through specific DNA repair pathways that include the BRCA pathway,^{7,17} whereas cancer cells with defects in the DNA damage response are more sensitive to platinum and die. Indeed, the results shown here suggest that alteration in *NBS1*

Table 3 Correlation of *NBS1* protein level and clinicopathological parameters

	Number of patients	<i>NBS1</i> expression		P-value
		Negative	Positive	
<i>FIGO stage</i>				
Early (I, II)	36	16	20	0.001
Advanced (III, IV)	104	13	91	
<i>Histology</i>				
Serous	82	11	71	0.008
Non-serous	58	22	36	
<i>Grade</i>				
1	23	14	9	0.001
2 and 3	117	16	101	
<i>Residual tumor</i>				
≤ 1 cm	73	28	45	0.021
> 1 cm	67	5	62	

Table 4 Univariate and multivariate analysis of prognostic factors in ovarian cancer patients for RFS

	Univariate analysis	Multivariate analysis	
	P-value	P-value	RR (95% CI)
<i>FIGO stage</i>			
Early (I, II) vs advanced (III, IV)	< 0.001	0.001	4.375 (2.920–6.555)
<i>Histology</i>			
Serous vs non-serous	< 0.001	0.045	0.363 (0.240–0.549)
<i>Grade</i>			
1 vs 2 and 3	0.004	0.012	2.269 (1.686–3.053)
<i>Residual tumor</i>			
≤ 1 cm vs > 1 cm	< 0.001	0.006	3.197 (1.905–5.364)
<i>NBS1 expression</i>			
Negative vs positive	0.005	0.035	1.929 (1.400–2.656)

Abbreviations: CI, confidence interval; RFS, recurrence-free survival; RR, relative risk.

expression level is associated with BRCAness, similar to *BRCA1* and *BRCA2* in *BRCA1*- or *BRCA2* mutated cancers. As EOCs that are sensitive to platinum are likely to have alterations in BRCA or the Fanconi Anemia pathway,^{18,19} the results shown here suggest that alteration in *NBS1* might reflect BRCAness, and that the BRCAness may be more prevalent in EOC than originally assumed, despite the small number of BRCA carriers in EOCs (10%–15%).

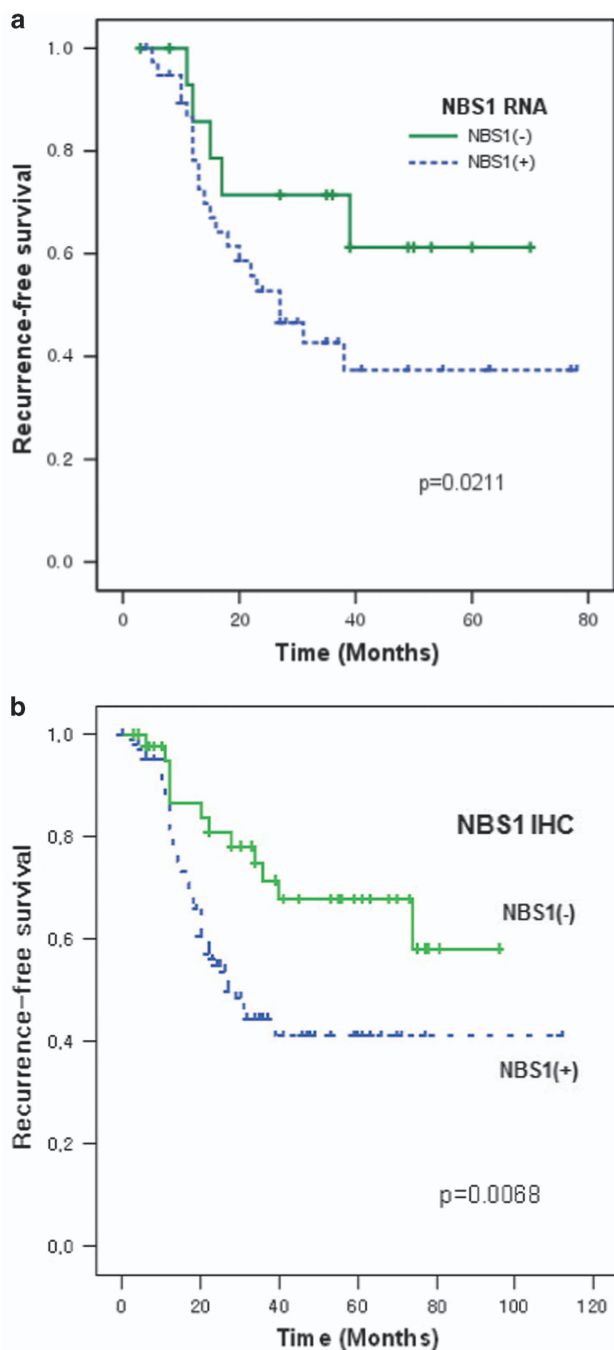


Figure 4 Recurrence-free survival (RFS) using the Kaplan–Meier method in relation to NBS1 levels. (a) Increased *NBS1* mRNA expression in the tumor shows poor RFS ($P=0.0211$). (b) NBS1 immunostaining score shows a significant correlation with RFS ($P=0.0068$).

To evaluate the impact of BRCAness genes related to HR in EOC, we selected representative BRCAness genes, including *BRCA1*, *BRCA2*, *BARD1*, *ATM*, *NBS1* and *RAD51*. Among them, the current study identified a novel association between NBS1 and EOC recurrence after treatment with cytoreductive surgery followed by platinum-based chemotherapy.

DSBs are repaired by non-homologous end-joining or HR. NBS1 is a critical component of the MRN complex (MRE11-RAD50-NBS1), which senses DSBs and has an essential role in processing DSBs before non-homologous end-joining or HR.^{20,21} Inheritance of *NBS1* mutation is the cause of the recessive genetic disorder Nijmegen breakage syndrome, manifested by elevated sensitivity to irradiation, chromosome instability and cancer predisposition.²² A high level of NBS1 expression is a prognostic marker in advanced head and neck squamous cell carcinoma and squamous cell carcinoma of the oral cavity.^{16,23} In lung cancer, a single-nucleotide polymorphism in NBS1 has been reported to modulate the occurrence of TP53 mutations and contribute to carcinogenesis.²⁴ In this study, we showed that *NBS1* expression has prognostic relevance in EOC, and that elevated levels of *NBS1* are associated with an advanced stage and grade of ovarian cancer.

Immunostaining of NBS1 protein by standard IHC techniques showed a strong association with tumor severity and metastatic death, strongly suggesting that IHC for NBS1 may be a clinically useful prognostic tool that can be applied at the time of initial surgical treatment. Mutations of the *NBS1* gene lead to multiple cancers including breast,²⁵ EOC,²⁶ bladder,²⁷ nasopharyngeal cancer²⁸ and leukemia.²⁹ Cancer polymorphism in *NBS1* may be a genetic modifier for the occurrence and aggression of cancer.

In conclusion, we suggest that NBS1 is a reliable prognostic marker for predicting RFS after initial treatment, and that assessing the levels of NBS1 can influence decisions in the adequate treatment of EOC. Furthermore, modulating the DNA repair pathway activity with PARP inhibitors may be a promising future approach to tailored therapy of EOC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

All experiments were approved by the Seoul National University Hospital IRB (1006-089-414). This study was supported by the Drug development program by NRF (2012 M3A9A 8052645), the Cancer Control program by the Korean Ministry of Health and Welfare (1220210) and the National Research Lab Program by NRF to HL (2013R1A2A1A01009184).

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