Western patients in previous trastuzumab emtansine trials, those adverse events recovered without special supportive treatment.

Key words: trastuzumab emtansine, HER2-positive, breast cancer, pharmacokinetics, safety

Introduction

Approximately 15–20% of human breast cancers have gene amplification or overexpression of human epidermal growth factor receptor 2 (HER2) (1). HER2 is a member of the epidermal growth factor receptor family of tyrosine kinase transmembrane receptors, and overexpression of HER2 is associated with aggressive tumor growth and poor clinical outcomes (1–3). Trastuzumab is a recombinant humanized monoclonal antibody targeted to the extracellular domain of HER2, and has significantly improved survival in patients with HER2-overexpressing metastatic breast cancer (MBC) (4, 5).

Trastuzumab emtansine (T-DM1) is a novel antibody–drug conjugate consisting of trastuzumab covalently bound via a thioether linker to DM1, a maytansinoid drug that binds to microtubules. Maytansine competes with vinca alkaloids for binding on the beta subunit of tubulin, with activity that is 20–100 times as potent as that of vincristine (6–8). In addition to its cytotoxic capabilities, T-DM1 retains the properties of trastuzumab, namely it inhibits HER2 cell proliferation signaling, and has antibody-dependent cellular cytotoxicity (9, 10).

In a Phase I study, the safety and tolerability of T-DM1 administered every 3 weeks was evaluated in HER2-positive MBC with prior trastuzumab treatment, and the maximum-tolerated dose (MTD) was determined to be 3.6 mg/kg with Grade 4 thrombocytopenia identified as the dose-limiting toxicity (DLT) (8). Based on results from that Phase I study, Phase II study was conducted with T-DM1 administered at 3.6 mg/kg every 3 weeks to patients with HER2-positive MBC who had received prior HER2-directed therapies; that Phase II study demonstrated an objective response rate of 25.9% (95% CI, 18.4–34.4%) (11). In another Phase II study, the ORR was 34.5% (95% CI, 26.1–43.9%) in patients with HER2-positive MBC who have previously received at least two lines of therapy including trastuzumab and lapatinib (12). A subsequent randomized Phase III study (EMILIA) compared T-DM1 with the combination of lapatinib plus capecitabine in patients with HER2-positive MBC who had previously been treated with trastuzumab and a taxane. Median progression-free survival was 9.6 months with T-DM1 versus 6.4 months with lapatinib plus capecitabine; $P < 0.001$), and overall survival at the second interim analysis crossed the stopping boundary for efficacy (30.9 months versus 25.1 months, $P < 0.001$) (13).

The primary objectives of this study were to determine the MTD and the recommend dose (RD) of T-DM1 in Japanese patients in whom the disease had progressed despite chemotherapy containing trastuzumab, the standard therapy for HER2-positive MBC.

Patients and methods

Patients

Eligible patients had histologically documented, inoperable advanced or recurrent HER2-positive breast cancer (centrally confirmed by immunohistochemistry or fluorescence *in situ* hybridization). All patients had a history of progression during, or up to 60 days after, treatment with a trastuzumab-containing regimen.

Additional eligibility criteria included the following: women aged 20 years or older; Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; adequate organ function [absolute neutrophil count, ≥1500/mm^3; platelet count, ≥100 000/mm^3; hemoglobin, >9.0 g/dl; total bilirubin, ≤1.5 mg/dl; hepatic transaminases, ≤2.5 times the upper limit of normal (ULN) (≤5 times the ULN if hepatic or bone metastases were presence); alkaline phosphatase (ALP), ≤2.5 times the ULN (≤5 times the ULN if bone metastases were present); serum creatinine, ≤1.5 mg/dl; and creatinine clearance, ≥60 ml/min]; no history of significant cardiac or hepatic disease; left ventricular ejection fraction, ≥50%; cumulative anthracycline dose, ≤360 mg/m^2 doxorubicin or equivalent (e.g. 720 mg/m^2 in the case of epirubicin); peripheral neuropathy, Grade ≤1; no treatment containing trastuzumab or lapatinib within 2 weeks of enrollment; no experimental therapy within 4 weeks of enrollment; no requirement for supplemental oxygen; and no history of Grade ≥3 hypersensitivity to trastuzumab. All patients provided written informed consent. This study was approved by the Institutional Review Board at each site according to local clinical guidelines and was performed as a registration-directed trial in accordance with the Good Clinical Practice guidelines laid down by the declaration of Helsinki, the study protocol and the revised Pharmaceutical Affairs Act in Japan.

Study design and treatment

T-DM1 was administered by intravenous infusion every 3 weeks. Premedications were not required prior to T-DM1 infusion. If an infusion related reaction occurred, pre-medications (acetaminophen and diphenhydramine hydrochloride) could be given with subsequent cycles per investigator discretion. The drug was infused over 90 min. If prior infusions were well tolerated, subsequent infusions were shortened to 30 min.

When this Phase I study was planned, two of three patients enrolled in the 4.8 mg/kg cohort experienced dose-limiting Grade 4 thrombocytopenia and MTD of 3.6 mg/kg every 3 weeks had been confirmed in the previous Phase I study. Therefore, a starting dose of 1.8 mg/kg, which was half of the previous Phase I RD (3.6 mg/kg), and three dose levels (Level 1, 1.8 mg/kg; Level 2, 2.4 mg/kg; Level 3, 3.6 mg/kg) were selected for this study. The continual reassessment method (CRM) with non-informative prior was used to determine the dose for each patient in this study (14). The planned maximum number of patients was 12. If the MTD was determined before the planned number of subjects was reached, enrollment was to be stopped. The MTD was defined as the dose at which the estimated probability of DLT was closest to 25%, which is the target probability of DLT. The probability of DLT at each dose was estimated using the one-parameter logistic model. The probability of DLT at each dose was estimated using the modified CRM.

DLTs were defined as any of the following adverse events (AEs) that occurred during the DLT observation period (21 days from the first infusion of the investigational product): (i) Grade ≥3 non-hematologic toxicity (excluding therapy diarrhea, nausea and vomiting manageable with standard support therapy); (ii) Grade ≥4 decreased platelet count; (iii) Grade ≥4 decreased hemoglobin; (iv) Grade ≥4 decreased absolute neutrophil count lasting for >4 days or accompanied by a fever of ≥38°C; (v) Grade ≥3 increased total

bilirubin or increased aspartate aminotransferase (AST), alanine aminotransferase (ALT) or ALP; or (vi) Grade ≥3 cardiotoxicity. Hematologic parameters and blood chemistry were tested on Days 1, 2, 3, 5, 8, 11, 15 and 19 in Cycle 1. The severity of the AE was assessed in accordance with the National Cancer Institute's Common Terminology Criteria for Adverse Events version 3.0 (15). Tumor responses were evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 (16).

Pharmacokinetic studies

Whole-blood samples (3.5 ml tube for T-DM1 and total trastuzumab in serum, and 2 ml heparinized tube for free DM1 in plasma) were drawn before infusion (pre) and at 30 min, 4, 24, 48, 96 h, and 7, 10, 14, 17/18 days (±6 h) after the end of infusion at Cycle 1; pre and 30 min after the end of infusion in subsequent cycles; and 4 h after the end of infusion at Cycle 3 and at termination of the study. Samples were inverted 5–10 times immediately, left 30 min at room temperature, then centrifuged at 1500–2000 *g* for 10–15 min, and aliquots of the resultant serum and plasma were stored at −70°C until analysis. Concentrations of T-DM1 and total trastuzumab in serum were quantitated using validated enzyme-linked immunosorbent assay methods by Genentech, Inc. (South San Francisco, CA, USA). Concentration of free DM1 in plasma was quantitated using validated liquid chromatography and tandem mass spectrometry by Quest Pharmaceutical Services (Groningen, the Netherlands). The lower limits of quantification were 40 ng/ml for T-DM1 in serum, 40 ng/ml for total trastuzumab in serum and 0.737 ng/ml for free DM1 in plasma.

Each patient's plasma concentration profiles of T-DM1, total trastuzumab and free DM1 were used to estimate the terminal half-life ($t_{1/2}$), area under the serum (plasma) concentration–time curve (AUC), total body clearance and volume of distribution (V_{ss}) by a non-compartment analysis using the Phoenix WinNonlin software (version 6.1, Pharsight Corp., CA, USA). The maximum concentration (C_{max}) of T-DM1, total trastuzumab and free DM1 was determined from the actual measured values. The AUC was calculated by the trapezoidal rule. The AUC_{inf} was obtained by summation of the AUC_{0-t} and the extrapolated area estimated by taking the ratio between the last measurable concentration and the apparent elimination rate constant. Regression analyses of each of individual C_{max}, AUC_{0-t}, and AUC_{inf} values versus the dose were performed to evaluate the pharmacokinetic linearity. SAS software (version 9.2, SAS Institute, Inc., NC, USA) was used for statistical analysis.

Whole-blood samples (5 ml tube containing EDTA-2Na) were collected prior to the first dose for analysis of genetic polymorphisms (FcγR, β1 tubulin). These were analyzed at Quest Diagnostics, Inc. (CA, USA).

Whole-blood samples (3.5 ml) for detection of antitherapeutic antibodies (ATA) were drawn at pre-dose in every cycle and at termination of the study. ATA was quantitated using validated enzyme-linked immunosorbent assay at Genentech, Inc.

Results

Patient characteristics

Ten patients were enrolled from three centers in Japan between September 2009 and July 2010, and all patients received at least two cycles of T-DM1 (median, 7 cycles; range, 2–26 cycles). Patient characteristics are shown in Table 1.

Of the 10 patients, 9 withdrew from this study but 1 patient was still continuing as of the data cutoff date (29 July 2011). Of the 9 patients withdrawn, 6 patients were withdrawn because of disease progression and 3 patients were withdrawn because of AEs (Grade 3 thrombocytopenia at 2.4 mg/kg, Grade 3 cholelithiasis at 2.4 mg/kg, and Grade 2 ALP increased at 3.6 mg/kg).

Table 1. Patient baseline demographics and disease characteristics ($N = 10$)

Characteristics	
Age, years	
Median	61.0
Range	36–76
ECOG PS (*n*)	
0	5
1	5
ER-positive and/or PgR-positive (*n*)	3
No. of prior metastatic chemotherapy regimens	
Median	4.5
Range	1–7
Time since last trastuzumab therapy, weeks	
Median	14.5
Range	2.7–54.9
Received prior anthracycline therapy (*n*)	8
Received prior taxane therapy (*n*)	10
No. of distinct metastatic sites (*n*)	
<3	7
≥3	3
Metastatic sites (*n*)	
Bone	2
Lung	4
Liver	4

ECOG, Eastern Cooperative Oncology Group; PS, performance status; ER, estrogen receptor; PgR, progesterone receptor.

Pharmacokinetic and immunogenicity analyses

Blood samples for pharmacokinetic analysis were available from all 10 enrolled patients in the first cycle. Serum concentration–time curves are shown in Figs. 1 and 2, and pharmacokinetic parameters for T-DM1 in serum are shown in Table 2. The serum T-DM1 in Cycle 1 showed a multiphasic elimination pattern, with peaks reached at 30 min and 4 h after the end of infusion, and rapid elimination from Day 1 to Day 2 after infusion with gradual elimination thereafter. Systemic clearance of T-DM1 was more rapid than clearance of total trastuzumab: at the MTD (3.6 mg/kg), clearance of T-DM1 was 10.6 ± 1.26 ml/day/kg and clearance of total trastuzumab was 5.45 ± 1.98 ml/day/kg. At the MTD, the terminal half-life of T-DM1 was 3.74 ± 1.15 days and terminal half-life of total trastuzumab was 6.47 ± 2.40 days. The AUC of free DM1 in plasma was <1/10 000 that of serum T-DM1 by mass and ~1/100 that of serum T-DM1 by molar equivalent. The pharmacokinetics of T-DM1 was linear in the range of 1.8–3.6 mg/kg.

Anti-T-DM1 antibodies were assessed for all enrolled patients. In the 3.6 mg/kg cohort, one patient was positive for anti-T-DM1 antibodies after the completion of Cycle 8. This patient, whose best response was partial response, experienced no Grade ≥3 adverse events.

Toxicities

At least one drug-related all grade AE was reported in each of the 10 patients. Types and incidences of AEs are listed in Table 3. AEs (all grades) observed in three or more patients were nausea and arthralgia ($n = 7$), fever ($n = 6$), fatigue and decreased appetite ($n = 5$), diarrhea,

malaise, headache, and rash ($n = 4$), and constipation, vomiting, myalgia, chills and cystitis ($n = 3$). There were no deaths during the study period. Drug-related alopecia and neuropathy were not reported.

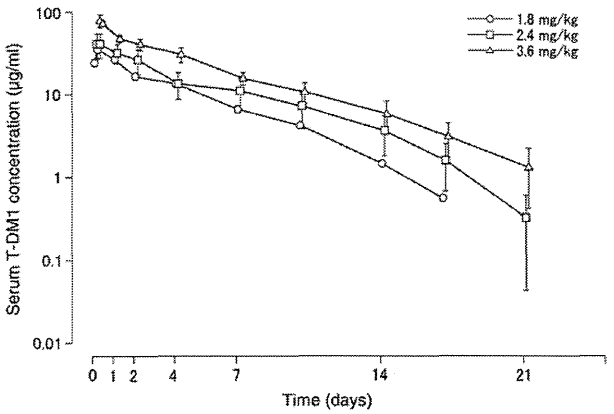

Figure 1. Pharmacokinetics of trastuzumab emtansine (T-DM1) by cohort. Mean concentration–time curves of T-DM1 in serum for doses of 1.8–3.6 mg/kg. Error bars show standard deviation. The measured values are displayed for the dose of 1.8 mg/kg, because there was only one patient.

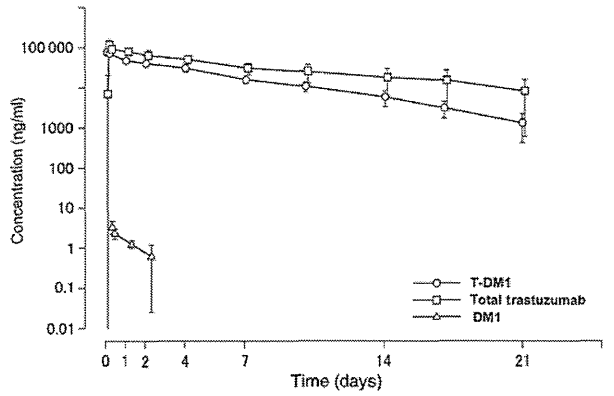

Figure 2. Pharmacokinetics of T-DM1, total trastuzumab, and free DM1 at MTD. Mean concentration–time curves of T-DM1 in serum, total trastuzumab in serum and free DM1 in plasma after the first dose of T-DM1 administered at MTD (3.6 mg/kg). Error bars show standard deviation.

Grade 3 or 4 AEs were observed in three of the four patients in the 2.4 mg/kg cohort (elevations of AST/ALT, cholelithiasis and thrombocytopenia, respectively). None of the five patients in the 3.6 mg/kg cohort experienced any Grade 3 or 4 AEs. Serious AEs occurred in two patients: one was cholelithiasis in the 2.4 mg/kg cohort, and the other was gastric ulcer hemorrhage in the 3.6 mg/kg cohort. AEs that resulted in discontinuation of treatment were thrombocytopenia ($n = 1$, 2.4 mg/kg cohort), cholelithiasis ($n = 1$, 2.4 mg/kg cohort), blood ALP increased ($n = 1$, 3.6 mg/kg cohort).

Dose modification due to elevation of AST/ALT was performed in one patient in the 2.4 mg/kg cohort. In five patients, treatment cycles were prolonged because of AEs (in the 2.4 mg/kg cohort, one patient with thrombocytopenia, one patient with neutropenia and elevation of serum amylase; in the 3.6 mg/kg cohort, one patient with thrombocytopenia, one patient with elevation of total bilirubin and gastric ulcer hemorrhage, and one patient with elevation of ALP).

Determination of MTD

The one patient who was treated at the 1.8 mg/kg dose level did not experience any grade ≥3 AE. Of the four patients in the 2.4 mg/kg cohort, one experienced Grade 3 elevation of AST/ALT that met the definition of DLT in the first cycle. This patient continued the therapy without delay by dose reduction. Of the five patients in the 3.6 mg/kg cohort, none experienced any Grade ≥3 AE. The MTD was determined to be 3.6 mg/kg at the point when the DLT evaluation period was completed in 10 patients because MTD would be unchanged even if subsequent two patients were enrolled.

Efficacy

The best overall response was determined in nine patients (one patient was still continuing as of the cutoff date, 29 July 2011). No complete response was observed. Partial response was observed in one patient in the 3.6 mg/kg cohort. Stable disease was observed in six patients (three in the 2.4 mg/kg cohort; three in the 3.6 mg/kg cohort), and progressive disease was observed in two patients had (one in the 1.8 mg/kg cohort; one in the 3.6 mg/kg cohort).

Discussion

In this Phase I study, we assessed the safety, tolerability, pharmacokinetics and efficacy of T-DM1 when intravenously infused every 3 weeks

Table 2. Pharmacokinetic parameters of T-DM1, total trastuzumab and DM1 in Cycle 1

Analyte	Dose (mg/kg)	No. of patients	C_{max} (μg/ml)		AUC_{last} (μg·day/ml)		AUC_{inf} (μg·day/ml)		$t_{1/2}$ (days)		V_{ss} (ml/kg)		Clearance (ml/day/kg)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
T-DM1	1.8	1	35.3		139		141		2.39		57.1		12.9	
T-DM1	2.4	4	43.4	15.2	203	69.9	204	70.5	2.88	0.317	67.6	20.3	13.4	6.34
T-DM1	3.6	5	82.0	10.0	338	36.2	346	41.1	3.74	1.15	59.1	6.62	10.6	1.26
TTmab	3.6	5	118	38.1	665	236	761	347	6.47	2.40	44.4	5.58	5.45	1.98
DM1	3.6	5	3.41	1.15	3.49	2.13	8.28	3.63	3.12	1.28	–		–	

T-DM1, trastuzumab–DM1; TTmab, total trastumzumab; C_{max}, maximum serum concentration; AUC_{last}, area under the serum concentration–time curve from time zero to the last measurable concentration; AUC_{inf}, area under the serum concentration–time curve from time zero extrapolated to infinity; $t_{1/2}$, terminal half-life; V_{ss}, volume of distribution, steady state; SD, standard deviation.

Table 3. Adverse events related to study drug reported in two or more of patients or at Grade ≥3

MedDRA System organ class preferred term for AE	ALL ($N = 10$)					1.8 mg/kg ($n = 1$)					2.4 mg/kg ($n = 4$)					3.6 mg/kg ($n = 5$)				
	Grade					Grade					Grade					Grade				
	All	1	2	3	4	All	1	2	3	4	All	1	2	3	4	All	1	2	3	4
No. of patients with at least one AE	10	10	7	3	–	1	1	1	–	–	4	4	2	3	–	5	5	4	–	–
Gastrointestinal disorders																				
Nausea	7	7	–	–	–	1	1	–	–	–	2	2	–	–	–	4	4	–	–	–
Diarrhea	4	4	–	–	–	–	–	–	–	–	1	1	–	–	–	3	3	–	–	–
Constipation	3	2	1	–	–	–	–	–	–	–	1	1	–	–	–	2	1	1	–	–
Vomiting	3	3	–	–	–	–	–	–	–	–	–	–	–	–	–	3	3	–	–	–
Musculoskeletal and connective tissue disorders																				
Arthralgia	7	6	1	–	–	1	1	–	–	–	3	3	–	–	–	3	2	1	–	–
Myalgia	3	3	–	–	–	–	–	–	–	–	1	1	–	–	–	2	2	–	–	–
Musculoskeletal pain	2	2	–	–	–	–	–	–	–	–	1	1	–	–	–	1	1	–	–	–
General disorders and administration site conditions																				
Fever	6	5	1	–	–	1	1	–	–	–	2	1	1	–	–	3	3	–	–	–
Fatigue	5	5	–	–	–	1	1	–	–	–	1	1	–	–	–	3	3	–	–	–
Malaise	4	3	1	–	–	–	–	–	–	–	1	1	–	–	–	3	2	1	–	–
Chills	3	2	1	–	–	1	1	–	–	–	2	1	1	–	–	–	–	–	–	–
Oedema peripheral	2	2	–	–	–	–	–	–	–	–	1	1	–	–	–	1	1	–	–	–
Infections and infestations																				
Cystitis	3	3	–	–	–	–	–	–	–	–	1	1	–	–	–	2	2	–	–	–
Investigations																				
Aspartate aminotransferase increased	2	–	1	1	–	–	–	–	–	–	2	–	1	1	–	–	–	–	–	–
Alanine aminotransferase increased	2	–	1	1	–	–	–	–	–	–	2	–	1	1	–	–	–	–	–	–
Blood alkaline phosphatase increased	2	1	1	–	–	–	–	–	–	–	1	1	–	–	–	1	–	1	–	–
Respiratory, thoracic and mediastinal disorders																				
Dyspnea	2	2	–	–	–	–	–	–	–	–	1	1	–	–	–	1	1	–	–	–
Oropharyngeal pain	2	2	–	–	–	–	–	–	–	–	1	1	–	–	–	1	1	–	–	–
Epistaxis	2	2	–	–	–	–	–	–	–	–	1	1	–	–	–	1	1	–	–	–
Rhinorrhea	2	2	–	–	–	–	–	–	–	–	1	1	–	–	–	1	1	–	–	–
Nervous system disorders																				
Headache	4	2	2	–	–	1	–	1	–	–	1	1	–	–	–	2	1	1	–	–
Cardiac disorders																				
Pericardial effusion	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–	2	2	–	–	–
Metabolism and nutrition disorders																				
Decreased appetite	5	4	1	–	–	–	–	–	–	–	1	–	1	–	–	4	4	–	–	–
Skin and subcutaneous tissue disorders																				
Rash	4	4	–	–	–	–	–	–	–	–	2	2	–	–	–	2	2	–	–	–
Blood and lymphatic system disorders																				
Thrombocytopenia	1	–	–	1	–	–	–	–	–	–	1	–	–	1	–	–	–	–	–	–
Hepatobiliary disorders																				
Cholelithiasis	1	–	–	1	–	–	–	–	–	–	1	–	–	1	–	–	–	–	–	–

AE, adverse event; MedDRA, medical dictionary for regulatory activities.

in Japanese patients with HER2-positive MBC. In the previous Western first-in-human study (TDM3569g), the MTD was determined to be 3.6 mg/kg (8). The MTD in Japanese patients was also estimated to be 3.6 mg/kg, and this dose was generally well tolerated. In generally, dose escalation, 3 + 3 design case, the patient can be enrolled in next cohort after DLT assessment of last patient at each cohort was completed and six patients are required to move to next cohort or determine MTD if a DLT is observed in the cohort. In this study, patients can be enrolled more seamlessly by using CRM, and therefore study period was shortened.

The pharmacokinetic profile of serum T-DM1 revealed moderate interindividual variability. It was concluded that the pharmacokinetics of serum T-DM1 is linear when the dose of T-DM1 is in the range of 1.8–3.6 mg/kg. Pharmacokinetic data do not suggest any apparent difference between Japanese patients and Western patients. The observed pharmacokinetics profile of serum T-DM1 in the 3.6 mg/kg cohort (Table 2) was similar to that seen in TDM3569g (C_{max}: 82.0 versus 74.3 μg/ml; AUC_{inf}: 346 versus 295.2 μg·day/ml; $t_{1/2}$: 3.74 versus 3.5 days; V_{ss}: 59.1 versus 60 ml/kg; clearance: 10.6 versus 12.9 ml/day/kg) (8).The Grade ≥3 AEs observed in this study were elevation of hepatic transaminases, thrombocytopenia and cholelithiasis. There were no cases presenting with clinically significant bleeding events. The most frequent AEs were nausea, arthralgia, fever, fatigue and decreased appetite (Table 3). Incidence of these common AEs was a little different from that of TDM3569g (nausea: 25.0%; arthralgia: 8.3%; fever: 8.3%; fatigue: 37.5%; decreased appetite: 0%) (8); however, the severity of these AEs was generally mild (none of them were Grade ≥3), and they were well managed by supportive treatments. Gastrointestinal toxicities, especially nausea, were common but mild (all events of nausea occurring in this study were Grade 1) and tended

to persist for 1–3 days; 5 of 7 events of nausea first occurred in Cycle 1 (two events occurred within 24 h from T-DM1 administration). These toxicities of T-DM1 were tolerable in Japanese patients. Hypokalemia was not observed in Japanese patients, whereas in TDM3569g and the Phase II study (TDM4258g and TDM4370g) in the USA, hypokalemia was reported in 4.2% (1 of 24 patients), 24.1% (27 of 112 patients) and 20.9% (23 of 110 patients), respectively. Hypokalemia was not associated with vomiting, diarrhea or diuretic use, and the mechanism of the hypokalemia was unclear (8, 11, 12).

S One of the mechanisms of transient thrombocytopenia and hepatotoxicity is thought to be internalization of T-DM1 into Fc-gamma receptor-bearing megakaryocytes and Kupffer cells with subsequent release of free DM1 or DM1-related hepatic injury due to the low levels of free DM1 (8). DM1 is a thiol-containing maytansinoid derived from the naturally occurring ester ansamitocin P-3. The related plant ester, maytansine, has been studied as a chemotherapeutic agent. Gastrointestinal toxicities (nausea, vomiting and diarrhea) are common and are dose-limiting toxicities. A pattern of transient, but also at times severe, hepatotoxicity has also been reported (17–22). In this study, free DM1 in plasma was detected in low levels. Concentration of free DM1 in plasma reached a peak at 30 min after infusion and decreased to below the limit of quantitation by Day 8 in the 3.6 mg/kg cohort (Fig. 2). Therefore, the cause of the common but mild gastrointestinal toxicities and also the reversible thrombocytopenia and hepatotoxicity seen in this study may be possibly attributed to transient and low levels of circulating DM1. Other mechanisms may contribute to the occurrence of the other AEs.

In conclusion, this is the first trial of T-DM1 in Japanese patients with HER2-positive MBC previously treated with chemotherapy containing trastuzumab. The results of this study indicate that T-DM1 may be dosed similarly in Japanese and Western patients, and that AEs are generally manageable. Transient thrombocytopenia and hepatotoxicity were observed in most cases. The different trends in severity and incidence rates of thrombocytopenia, hepatotoxicity and hypokalemia are expected to be verified by the results of the Japanese Phase II study (JO22997) evaluating the efficacy and safety of T-DM1 in Japanese patients with heavily pre-treated HER2-positive MBC.

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Conflict of interest statement

Kenjiro Aogi, Hiroji Iwata and Chikako Shimizu have received speaking fee from Chugai. Yasuhiro Fujiwara has received speaking fees from Astra Zeneka, Eisai, Kyowa Hakko Kirin, GlaxoSmithKline, Sanofi-Aventis, Daiichi Sankyo, Taiho, Takeda, Chugai, Eli Lilly, Novartis, Bristol-Myers and NEC Corporation. Yuriko Igawa, Takashi Asakawa and Mari Matsubara are employees of Chugai Pharmaceutical Co., Ltd. All remaining authors have declared no conflict of interest.

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Appendix

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特　集　血管新生を標的としたがん治療の現況と展望

血管新生を標的とした乳がん治療の現状と展望

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Anti-Angiogenic Therapy in Breast Cancer: Ryota Tanaka and Kenji Tamura (*Division of Breast and Medical Oncology, National Cancer Center Hospital*)

Summary

Angiogenesis is important for tumor growth and breast cancer development. Bevacizumab is a humanized monoclonal antibody that targets vascular endothelial growth factor (VEGF)-A, and it is the only anti-angiogenic agent approved for breast cancer treatment in Japan. In this article, we discuss the efficacy, tolerability, and potential future refinements for the use of bevacizumab. **Key words**: Anti-angiogenesis therapy, Breast cancer, VEGF, Tyrosine kinase inhibitors, **Corresponding author**: Ryota Tanaka, Division of Breast and Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

要旨　血管新生は乳がんの増殖や発展に重要な役割を果たしている。血管内皮細胞増殖因子VEGF-Aに対するヒト化モノクローナルIgG抗体であるbevacizumab（Avastin）は，本邦で唯一使用が承認されている血管新生阻害剤である。本稿では，bevacizumabを中心に血管新生阻害剤の乳がん治療での現状と展望について述べる。

はじめに

血管内皮細胞増殖因子（vascular endothelial growth factor: VEGF）は，腫瘍の血管新生により酸素と栄養が補給されてがんの増殖を引き起こす鍵となる分子である。VEGFを介した血管新生阻害標的治療には，VEGF-Aに対するヒト化モノクローナルIgG抗体であるbevacizumab（Avastin）や，VEGFRを含む血管新生シグナルを阻害するマルチキナーゼ阻害薬（VEGFR-TKIs: sorafenib, sunitinib, vandetanib, pazopanib, axitinibなど）が用いられる。

Ⅰ．治療の現状

1．進行性乳がん

これまでに行われた臨床試験のメタ解析では，化学療法へのbevacizumabの上乗せは，無増悪生存期間（progression-free survival: PFS）や奏効率（overall response rate: ORR）を改善することを示した。しかしながら，がん治療の効果を計るgold standardである全生存期間（overall survival: OS）延長の証明はなく，bevacizumabの上乗せによる毒性の発現頻度は増加した[1-3]。また，VEGFR-TKIsの上乗せを評価した臨床試験のメタ解析では，ORRの改善は認めたものの，PFS，OSのいずれにも延長は証明されず，臨床的有用性を示すには至っていない[4]。したがって，本稿ではbevacizumabの現状と展望につき取り上げる。

bevacizumabは，腫瘍から新生した血管の内皮細胞をアポトーシスさせることで血管を消退させ，内皮細胞の増殖や分化を阻止することで新生血管の形成を阻害している[5]。前臨床試験で，血管新生は乳がんの腫瘍浸潤や転移に関し中心的な役割をもっており，VEGF阻害が血管新生，増殖および転移を抑制することが示されている[6,7]。第Ⅰ/Ⅱ相試験では既治療の転移性乳がんに対し，3, 10, 20 mg/kgのbevacizumab単独投与を行った結果，ORR 9.3%，22週目での疾患制御率17%であり，10 mg/kgが推奨された[8]。

2．HER2陰性の再発/転移性乳がんへの一次治療

E2100試験（n=685）[9]では，paclitaxelとbevacizu-

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mab 併用群は paclitaxel 単独投与群に比べ，PFS の延長（median 11.4 か月 vs 5.8 か月）と ORR の改善（48.0% vs 23.4%，p=0.0001）を有意に認めた[10]。AVADO 試験（n=736）は docetaxel に対する bevacizumab（7.5, 15 mg/kg）上乗せの有効性と安全性を検証した試験であり，bevacizumab（15 mg/kg）群が placebo 群と比較して PFS を 1.9 か月延長した（p=0.06）。ORR も bevacizumab 上乗せ群が placebo 群よりも高かった（placebo 群 46% vs bevacizumab 7.5 mg/kg 群 55%，p=0.07，bevacizumab 15 mg/kg 群 64%，p<0.001）[11]。

RIBBON-1 試験（n=1,237）では，capecitabine, taxane-based, anthracycline-based chemotherapy に対する bevacizumab の併用効果の検証が行われた。各々の bevacizumab-chemotherapy 群は placebo-chemotherapy 群と比較して PFS の延長を認めた。最も顕著であったのが capecitabine 併用群であり，PFS は 5.7 か月から 8.6 か月へと延長した（p=0.0002）。また，ORR も各々の bevacizumab-chemotherapy 群のほうが高かった。しかしながら OS の有意な延長はなかった[12]。ATHENA 試験（n=2,251）では taxane-based chemotherapy と bevacizumab の併用効果が検証された。治療期間の中央値は bevacizumab 併用群 6.2 か月，化学療法単独群が 4.2 か月であった。intent-to-treat（ITT）population 解析では，無増悪期間（time to progression: TTP）9.5 か月 [95% confidence interval (CI): 9.1-9.9]，ORR 52%であった[13]。

3．HER2 陰性の再発/転移性乳がんへの二次治療

RIBBON-2 試験（n=684）は，二次治療での bevacizumab の化学療法との併用に対する有効性と安全性を検証した。化学療法と bevacizumab の併用は PFS を 5.1 か月から 7.2 か月に延長し，死亡リスクを 22%減少させた（HR: 0.78，95% CI: 0.64-0.93，p=0.0072）。ORR は併用群 39.5%に対し placebo 群 29.6%と併用群のほうが高い傾向にあったが，有意差はなかった[14]。AVF2119g 試験は capecitabine と bevacizumab との併用効果を検証したが，主要評価項目である PFS の延長を認めなかった（bevacizumab/capecitabine 併用群 4.9 か月 vs capecitabine 単独投与群 4.2 か月，HR: 0.98）[15]。

4．HER2 陽性の再発/転移性乳がんへの一次治療

AVEREL 試験（n=424）は trastuzumab/docetaxel（TH）への bevacizumab（B）の併用効果を検証する目的で行われた。investigator-assessed PFS（BTH 群 16.5 か月 vs TH 群 13.7 か月，HR: 0.82，95% CI: 0.65-1.02，p=0.08），ORR（BTH 群 74% vs TH 群 70%，p=0.3492），OS（HR: 1.01，95% CI: 0.74-1.38，p=0.95）であり，bevacizumab 併用による有意な効果は認めなかった[16]。

Ⅱ．早期乳がん

1．術前化学療法

HER2 陰性患者を対象にして bevacizumab（15 mg/kg）の上乗せ効果を検証した二つのランダム化比較第Ⅲ相試験が存在する。GeparQuinto 試験（n=1,948）[17]では，taxane，anthracycline への bevacizumab の上乗せは主要評価項目である pCR 率を上昇させなかった。また，乳房温存切除割合を上昇させなかった[17,18]。ただし，subpopulation である triple negative breast cancer（TNBC: HER2-negative，estrogen-/progesterone-receptor negative）群（n=663）では，pCR 率は上昇した（27.9% vs 39.3%，p=0.003）[19]。NSABP B-40 試験（n=1,206）[20]では，標準化学療法への bevacizumab の上乗せにより pCR 率は上昇した（28.4% vs 34.5%，p=0.02）。そして，それはホルモン陽性群で顕著であった（15.1% vs 23.2%，p=0.007）。このように，術前化学療法では bevacizumab の併用により pCR 率が上昇した患者群を探索する試みが行われている。ただし，最近では乳がんのサブタイプによっては pCR 率が長期予後のよい代替指標とならないという報告もあり[21]，薬物の上乗せにより pCR 率が上昇したとしても，OS は改善しないかもしれない。また，併用により有害事象（好中球減少，手足症候群，口内炎，高血圧）の割合は増加した。TNBC は乳がんを生物学的特徴で分類した時の一つの分子学的サブタイプであり，他のサブタイプと比較して再発リスクが高く，予後も不良であることが特徴である[22]。

2．術後化学療法

BEATRICE 試験（n=2,591）[23]は手術可能な HER2 陰性浸潤がん患者を対象に行われ，主要評価項目を invasive disease-free survival（IDFS）として，術後化学療法への上乗せ効果を検証したが，上乗せ効果はなかった [chemotherapy alone vs bevacizumab 16% vs 14%，HR=0.87（0.72-1.07），p=0.18]。死亡例が 200 となった時の OS にも差はなかった [HR=0.84（0.64-1.12），p=0.23]。一方で試験中止となる有害事象の発生率は bevacizumab 上乗せ群で高かった [256（20%）vs 30（2%）]。そして，bevacizumab は術後補助療法として推奨されない結論に至った。

Ⅲ．治療の展望

bevacizumab をはじめとした血管新生阻害剤をより効果的に使用するためには，腫瘍の HER2 蛋白発現ないし遺伝子増幅例に trastuzumab を使用するのと同様に，治療効果予測因子などの治療に役立つ指標（バイオマー

カー）を見つけ，それによる患者選択が重要となるであろう[24,25]。AVADO試験，AVEREL試験では，血漿中のVEGF-Aの探索的な解析が行われ，base-lineでVEGF-A高値群は低値群と比較してbevacizumabの併用効果が高い傾向にあった（統計学的有意差はなし）。GO25632（MERiDIAN）試験では，血漿中のVEGF-A値を層別化して臨床試験が行われる。

また，HER2やホルモン受容体のstatusにより治療効果の高い患者群を特定し，対象患者を層別化した臨床試験が現在進行中であり，ここではすでに行われた臨床試験サブグループ解析を解説する。

1．サブグループ解析

1）TNBC患者

E2100試験，AVADO試験，RIBBON-1試験でのTNBC患者のメタ解析（bevacizumab-chemotherapy群n=363，chemotherapy alone群n=258）では，bevacizumab-chemotherapy群において，PFSの有意な延長効果を認め（8.1か月 vs 5.4か月，HR: 0.63，p<0.0001），ORRも有意に改善した（42% vs 23%，p<0.0001）[26]。

ATHENA試験での探索的なサブグループ解析では，paclitaxel/bevacizumabの併用が有用とされ［TTP 7.2か月（95% CI: 6.6-7.8），ORR 49%，CR率10%］，OSは全体で25.2か月，TNBC subgroup 18.3か月（95% CI: 16.4-19.7），non-TNBC subgroup 27.3か月であった[27]。

RIBBON-2試験でのサブグループ解析では，bevacizumabを含まない化学療法の一次治療を行った後に進行したTNBC患者への二次治療で，bevacizumabの併用は効果を示し（6.0か月 vs 2.7か月，HR: 0.494，95% CI: 0.33-0.74，p=0.0006），ORRも有意に改善した（41% vs 18%，p=0.0006）。また，少数例でデータも十分でなかったがOSを延長する傾向を示した（17.9か月 vs 12.6か月，HR: 0.624，95% CI: 0.39-1.007，p=0.0534）[28]。

2）HER2陰性の高齢の転移性乳がん患者

AVADO試験には，127人のHER2陰性高齢患者（≥65歳）が含まれていた[29]。bevacizumab投与によりPFSは延長した。効果は15 mg/kg投与群のほうが高い傾向にあった［10.1か月 vs 7.7か月（7.5 mg/kg群），HR: 0.68，95% CI: 0.428-1.076］。ORRもbevacizumab併用群がdocetaxel単独投与群に比べ高かった（50% vs 45%）。安全性評価は全患者と比較しても同等であったが，bevacizumab投与により動脈塞栓症の発症リスクがあることから，65歳以上の高齢者には低用量アスピリンの併用が推奨された[30]。

ATHENA試験での高齢者（≥70歳）のsub-populationのうち（n=175），46%の患者がpaclitaxelとbevacizumabの併用治療を受けた。TTPの中央値10.4か月，ORRは42%であった。高齢者群のOSは20.4か月であった。有害事象のうち，高齢者では，若年者と比較して高血圧と蛋白尿の頻度が高かった（Grade≥3高血圧: 6.9% vs 4.2%，Grade≥3蛋白尿: 4.0% vs 1.5%）[31]。

3）Taxane既治療のHER2陰性患者に対する二次治療

E2100試験とAVADO試験でtaxane既治療へtaxaneの再投与を行ったsub-population解析では（n=311），bevacizumabとの併用により効果が示唆された。PFSはbevacizumab併用群が10.7か月であったのに対し，taxane単独投与群は6.2か月であった（HR: 0.533，p=0.0001）。ORRもbevacizumab併用群のほうが高かった（49% vs 27%，p<0.005）。ただし，E2100試験では，taxane既治療の有無での割り付けがされておらず，AVADO試験では，taxane既治療例は少数（n=77）であった[32]。

おわりに

これまで行われた臨床試験からは，マルチキナーゼ阻害薬が臨床的有用性を示すには至っていない。bevacizumabの化学療法への上乗せは，進行乳がんにおいてPFSやORRを改善することを示した。早期乳がんでは，術前化学療法との併用でpCR率の上昇が報告された試験が存在する。しかしながら，いずれもがん治療の効果を計るgold standardであるOSの延長を直接証明してはいない。また，毒性の発現頻度はbevacizumab併用により増加した。今後，血管新生阻害剤をより効果的に使用し，OSの延長効果とQOL改善の効果を示すためには，治療効果予測因子などの治療に役立つ指標（バイオマーカー）の同定，HER2やホルモン受容体のstatusにより治療効果の高い患者群を特定する必要があり，現在それらを層別化した臨床試験が進行中である。

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BRIEF REPORTS

Desmoplastic Small Round Cell Tumor With Sphere-like Clusters Mimicking Adenocarcinoma

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Desmoplastic small round cell tumor (DSRCT) is a rare and aggressive neoplasm that predominantly affects young men. DSRCT often presents as multiple nodules on the serosal surface and is histologically categorized as a small round cell tumor. However, the cytological spectrum of DSRCT is not fully understood because of its rarity. Here, we report an unusual case of DSRCT that showed spheres of cells without stromal cores in pleural fluid cytology material, a finding that is typically associated with metastatic adenocarcinoma and mesothelioma. The specimen from a simultaneous needle biopsy showed the classic histology of DSRCT, comprising nests of small round cells set in desmoplasia. The diagnosis of DSRCT was further supported by immunohistochemical coexpression of cytokeratin and desmin, as well as Ewing sarcoma breakpoint region 1 gene rearrangement, which was determined by fluorescence in situ hybridization. The unusual cytological finding in this case illustrates a potential pitfall of the cytological diagnosis of pleural fluid or ascites. DSRCT should not be excluded from the differential diagnosis when sphere-like round cell clusters are observed in pleural or abdominal effusion, particularly in young male patients. Diagn. Cytopathol. 2015;43:214–217.

Key Words: desmoplastic small round cell tumor; pleural effusion; cytology

Desmoplastic small round cell tumor (DSRCT) is a rare neoplasm, first described in 1989 by Gerald and Rosai.[1] It predominantly affects young men, with a peak incidence in the third decade of life.[2] The tumor mainly presents as multiple nodules on the peritoneum and tends to spread diffusely over the peritoneal surface.[2] Various other primary sites have been reported, such as the pleura, paratesticular area, ovary, lung, intracranial area, head and neck areas, pancreas, and kidney.[2–4] DSRCT is an aggressive neoplasm, and most patients die within 2 years of diagnosis.[4]

Histologically, DSRCT is typically composed of small round cells with hyperchromatic nuclei and scant cytoplasm. The cells are arranged within a desmoplastic stroma in small nests or single files. However, approximately one-third of the tumors show various histological features, such as gland-like structure, signet ring-like morphology, rhabdoid cells, and spindling.[2,5] Immunohistochemistry plays an important role in the diagnosis of DSRCT; cytokeratin, and dot-like desmin expression are characteristic.[2] Genetically, DSRCTs have a specific chromosomal translocation, t(11;22)(p13;q12), which results in the fusion of the Ewing sarcoma breakpoint region 1 (*EWSR1*)gene and the Wilms' tumor gene (*WT1*).[2,4]

Because of the rarity of DSRCT, its cytological features have been described for only approximately 30 cases, mostly in case reports and small series.[3,6–19] A typical smear of fine-needle aspiration biopsy material shows tumors cells in loosely cohesive three-dimensional clusters with occasional isolated cells. Some clusters may have a rosette-like appearance.[8,11,16,19] Tumor cells demonstrate high nuclear-cytoplasmic ratios, round-to-oval or irregular nuclei with granular chromatin, and inconspicuous nucleoli. Here, we report a case of DSRCT that showed unique sphere-like three-dimensional clusters in the cytology specimen. We emphasize that this cytological finding is a potential diagnostic pitfall because it is commonly interpreted as suggesting metastatic adenocarcinoma or mesothelioma.

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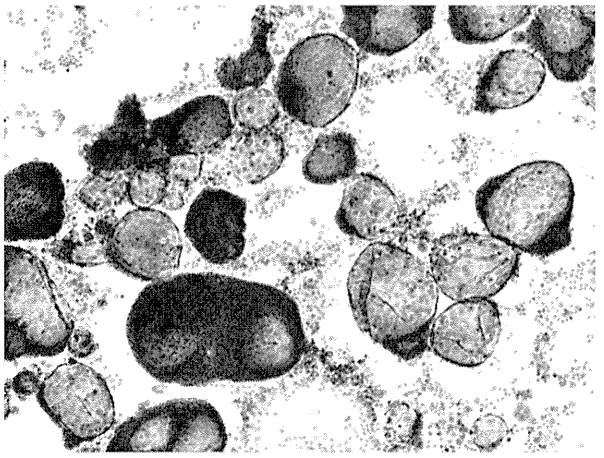

Fig. 1. At low magnification of the pleural fluid smear, it is apparent that tumor cells are organized in numerous sphere-like three-dimensional clusters without stromal cores. Tumor cells are arranged in monolayered sheets surrounding the central vacant space (Papanicolaou stain, 100×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

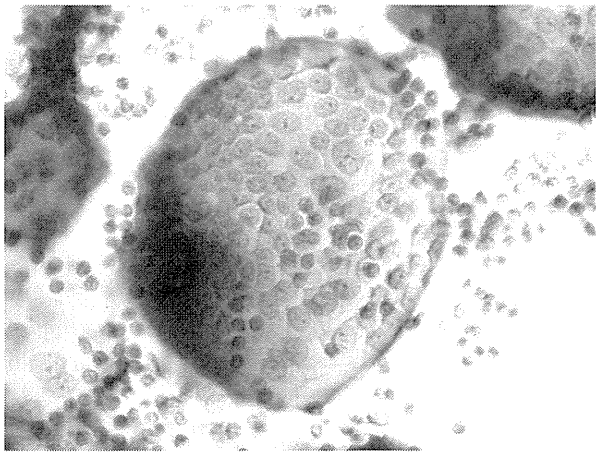

Fig. 2. Higher magnification of the pleural fluid smear reveals that tumor cells have round-to-oval nuclei with finely granular chromatin, inconspicuous nucleoli, and scant cytoplasm (Papanicolaou stain, 400×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Case Report

Presentation and Course

A 30-year-old man reported recent weight loss (from 64 to 60 kg) and night sweating. He had no personal or familial history of malignancy. A computed tomography (CT) scan revealed right pleural and abdominal effusion, multiple tumor masses in the thoracic and abdominal cavities, and many nodules in both lungs and in the liver. The radiographic findings were consistent with the dissemination of a malignant neoplasm, but the CT scan did not reveal any obvious primary site. Thoracentesis, abdominocentesis, and a CT-guided needle biopsy of the pleural mass were performed. The patient continued to deteriorate, experienced multiorgan failure, and died 17 months after he first experienced weight loss. An autopsy was not performed.

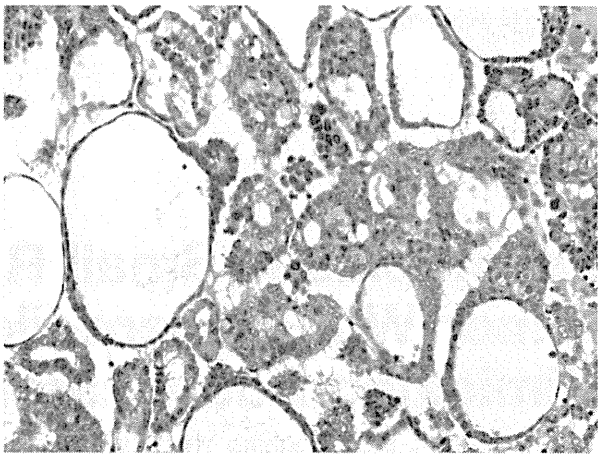

Fig. 3. A cell block specimen of the pleural fluid reveals that the tumor cells form numerous hollow ring-like structures (hematoxylin and eosin stain, 200×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Cytological and Cell Block Findings

Papanicolaou staining of the pleural fluid revealed a moderate to high cellularity (Fig. 1). Tumor cells were arranged in three-dimensional clusters, most of which were less than 0.1 mm in diameter. Isolated tumor cells were rare. The clusters did not have stromal cores, and monolayered sheets of tumor cells surrounded the central vacant space. Tumor cells were 2 to 4 times the size of lymphocytes and had enlarged round-to-oval nuclei (Fig. 2). The nuclei contained finely granular chromatin and had regular thin nuclear membranes, which appeared slightly wrinkled in some cells. Many of the nuclei had one small nucleolus. The cytoplasm was pale blue, slightly ill defined, and scarce. The nuclear-to-cytoplasmic ratio was high. Mitoses were readily noted. Unequivocal gland or rosette-like formations were not observed. The background included a moderate number of lymphocytes with a few mesothelial cells and macrophages. Necrotic debris was not seen.

A cell block of pleural fluid showed similar findings to those in the smear (Fig. 3). Tumor cells formed hollow ring-like structures. They were arranged in small nests that surrounded vacant centers. They had enlarged nuclei with slightly granular chromatin and a single small nucleolus. The background contained moderate quantities of lymphocytes and macrophages. No necrotic debris was found.

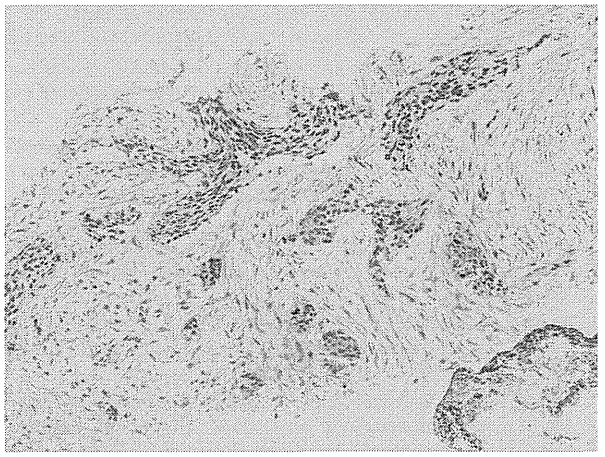

Fig. 4. In the histological specimen, small round cells form well-defined nests within a desmoplastic stroma. Tumor cells have small hyperchromatic nuclei and scant cytoplasm. This finding is typical of desmoplastic small round cell tumor (hematoxylin and eosin stain, 100×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

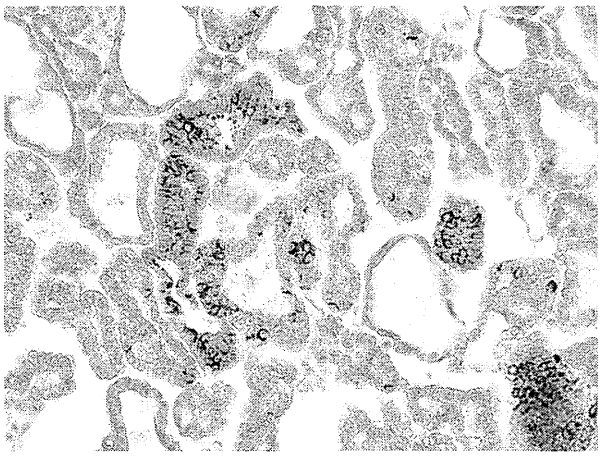

Fig. 5. Immunohistochemical examination of the cell block specimen reveals that the tumor cells show focal reactivity for desmin, partially in a dot-like pattern (immunoperoxidase, 400×). Tumor cells also show diffuse positivity for pan cytokeratin AE1/AE3 (data not shown). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Histological, Immunohistochemical, and Cytogenetic Findings

A CT-guided needle biopsy of the pleural mass revealed small round tumor cells forming discrete nests in a desmoplastic stroma (Fig. 4). Tumor cells showed slight pleomorphism. Unlike the tumor cells in the cell block specimen, those in the biopsy specimen did not form hollow ring-like arrangements. Immunohistochemical studies performed on the cell block and needle biopsy specimens showed identical results in both specimens. The tumor was positive for pan cytokeratin AE1/AE3, desmin, and vimentin. In the cell block specimen, desmin showed focal dot-like reactivity (Fig. 5). Immunostaining for Wilms' Tumor antigen 1 (WT1, to the N-terminus portion), thyroid transcription factor-1, and cytokeratin 5/6 were negative. A fluorescence in situ hybridization (FISH) analysis performed on the cell block specimen revealed *EWSR1* gene rearrangement (Fig. 6).

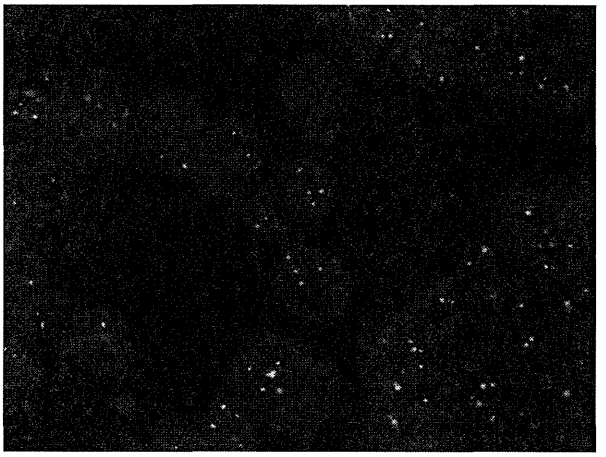

Fig. 6. A fluorescence in situ hybridization analysis of cell block specimen shows separation of the green and red signals, indicating break apart rearrangement of the Ewing sarcoma breakpoint region 1 gene (1000×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Discussion

In this report, we described a case of DSRCT that showed sphere-like clusters, a finding that has not been reported previously for this disease. These clusters had a typical immunoprofile and *EWSR1* rearrangement (as assessed by FISH), confirming that they represent an unusual growth pattern of DSRCT. This cytological finding sharply contrasts with classic cytological descriptions of DSRCT, in which loosely cohesive clusters are observed along with occasional isolated cells. Instead, we observed tightly packed, round-to-oval spheres without stromal cores, which closely resembled the so-called "proliferation spheres"[20–22] that have been reported in effusions from patients with metastatic breast carcinoma, metastatic ovarian carcinoma, epithelioid mesothelioma, and poorly differentiated small cell carcinoma.[20–22]

The morphogenic mechanism of the proliferation sphere-like clusters is not clear in this case. There are two hypothesises. One hypothesis is that they could be associated with tumor floating in the effusion. Indeed, in carcinomas, proliferation spheres are generally believed to form when tumor cells exfoliate into a cavity, reflecting the ability of malignant cells to proliferate in the nutrient-rich effusion.[21] The other is that the presence of proliferation sphere-like clusters in DSRCT may represent

yet another facet of the histological diversity that is characteristic of this tumor.[5] One limitation of our report was that there was no excisional biopsy or autopsy performed for precise histologic correlation. However, as there was no gland component in the needle biopsy specimen, we still believe that the former hypothesis is correct.

The differential diagnosis of multiple peritoneal or pleural masses is broad. In older patients, it should certainly include carcinomatosis, lymphomatosis, and malignant mesotheliomas, along with various infectious etiologies. For young male patients, we suggest that the differential diagnoses also include DSRCT despite its overall rarity,[23] because DSRCT has a distinctive clinical course that requires a specific management plan.

In conclusion, we have described a case of DSRCT with previously unreported proliferation sphere-like clusters in the cytology specimen. Although proliferation spheres are classically associated with carcinomas or mesotheliomas, rare cases of DSRCT may present clusters that have a similar appearance. This diagnostic pitfall should be particularly remembered when diagnosing young patients who have effusion with abdominal masses.

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BMC Cancer

RESEARCH ARTICLE Open Access

Nucleostemin expression in invasive breast cancer

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Abstract

Background: Recently, the cancer stem cell hypothesis has become widely accepted. Cancer stem cells are thought to possess the ability to undergo self-renewal and differentiation, similar to normal stem cells. Nucleostemin (NS), initially cloned from rat neural stem cells, binds to various proteins, including p53, in the nucleus and is thought to be a key molecule for stemness. NS is expressed in various types of cancers; therefore, its role in cancer pathogenesis is thought to be important. This study was conducted to clarify the clinicopathological and prognostic impact of NS in invasive breast cancers.

Method: The correlation between NS immunoreactivity and clinicopathological parameters was examined in 220 consecutive surgically resected invasive breast cancer tissue samples by using tissue microarrays. The presence of nuclear NS and p53 immunoreactivity in 10% or more of cancer cells was considered as a positive result.

Results: Among the 220 patients, 154 were hormone-receptor (HR)-positive, 22 HER2-positive/HR-negative, and 44 HR-negative/HER2-negative. One hundred and forty-two tumors (64.5%) showed NS positivity, and this positivity was significantly correlated with estrogen receptor (ER) ($P = 0.050$), human epidermal growth factor receptor 2 (HER2) ($P = 0.021$), and p53 ($P = 0.031$) positivity. The patients with NS-positive tumors showed significantly shorter disease-free survival than those with NS-negative tumors. Furthermore, the patient group with NS- and p53-positive tumors showed significantly poorer prognosis than other patient groups. Multivariate analysis showed that NS status was an independent prognostic indicator.

Conclusions: NS may play a significant role in the determination of breast cancer progression in association with p53 alterations. The NS status of patients with luminal and HER2 type breast cancers may be a useful prognostic marker.

Background

Breast cancer is one of the most prevalent diseases worldwide. While most patients with early breast cancers are cured with surgically resection followed by appropriate adjuvant drug and radiation therapy, approximately 30% of these patients experience relapse and develop metastatic disease [1]. In this metastatic stage, tumor cells frequently acquire resistance to various drugs during intensive systemic therapies, and eventually their aggressiveness and growth become uncontrollable. Less than 5% of patients with distant metastatic tumors live for 5 years [2]. Therefore, identification of potential targets with the aim of developing interventional drugs is an important area of research.

The hypothesis that various types of cancers, including breast cancer, are generated by a limited number of cancer stem cells has been widely accepted recently [3]. Cancer stem cells, like normal stem cells, are thought to have two important characteristics: the ability to undergo self-renewal and the ability to undergo differentiation into different cell types [4]. Furthermore, these cells are thought to be inherently resistant to various therapeutic drugs, making the eradication of tumors containing cancer stem cells with the use of the current treatment protocols difficult [5]. To overcome these obstacles, the development of new therapeutic strategies to target cancer stem cells is essential for the management of breast cancer.

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Nucleostemin (NS) is thought to be a key molecule for maintaining "stemness" [6]. NS was initially cloned from rat neural stem cells and was found to contain two GTP-binding motifs and an N-terminal basic domain, which is essential for binding to p53 [6]. NS accumulates mainly in the nucleoli and moves to the nucleoplasm after binding with GTP. Interaction of NS with a multitude of proteins in the nucleoplasm, including p53, may play a significant role in self-renewal, cell cycle regulation, apoptosis, and cell proliferation [7].

NS is expressed in central nervous system stem cells, embryonic stem cells, and primitive cells in the bone marrow and testes [6]. Furthermore, various types of cancers, including the following, have been reported to express NS: squamous cell carcinomas of the uterine cervix; head, neck, esophagus, and renal cell carcinomas; and prostate cancer [8-13]. Moreover, recent evidence indicates that NS is involved in maintaining cancer stem cells [14,15]. These findings suggest that NS may also play an important role in cancer pathogenesis as well as in cancer stem cell maintenance. However, no clinical study has investigated the role of NS in breast cancer.

If NS is expressed in breast cancer stem cells and its expression is correlated with disease progression in breast cancer, it may serve as a powerful prognostic marker for clinical use. To test this hypothesis, we investigated the expression of NS in surgically resected invasive breast cancer specimens from 220 patients by using immunohistochemistry. Furthermore, we examined the prognostic implication of the combination status of NS and p53 and the significance of NS expression status among the three biological subtypes of breast tumors: (a) hormone-receptor (HR) positive (luminal type); (b) human epidermal growth factor receptor 2 (HER2) positive (HER2 type); and (c) HR negative and HER2 negative (triple negative).

Methods

Patients and tumor specimens

The patient cohort used in the present study was the same as the cohort reported in our previous study [16]. Briefly, formalin-fixed paraffin-embedded tissue blocks of invasive breast cancer specimens from 220 consecutive patients were used to construct tissue microarrays (TMAs). All patients with unilateral invasive breast carcinoma underwent mastectomy or breast-conserving surgery at the National Defense Medical College (NDMC) Hospital, Tokorozawa, Japan from 1995 through 1999. These patients had a median follow-up of 74 months after surgery (range, 1–151 months), during which 58 patients experienced relapse. Of the 220 patients, 218 were female patients and 2 were male patients; 101 (45.9%) patients had lymph node metastasis and 8 (3.6%) had distant metastasis at the time of breast cancer diagnosis. In most cases, patients with hormone receptor-positive tumors at the time of diagnosis were prescribed adjuvant endocrine therapy (e.g., tamoxifen, toremifene, fadrozole, or LHRH analogues) for two or more years. The patients with a large tumor and/or four or more lymph node metastases received one of the following adjuvant chemotherapy regimens: cyclophosphamide-epirubicin-5-fluorouracil (CEF), cyclophosphamide-adriamycin-5-fluorouracil (CAF), cyclophosphamide-methotrexate-5-fluorouracil (CMF), and oral fluoropyrimidines. Detailed patient and disease characteristics are documented in Table 1. Clinicopathological data were retrospectively obtained from medical records [16].

This study was approved by the Medical Ethical Committee of National Defense Medical College and by the Institutional Review Board of National Cancer Center.

Tissue microarray construction

We constructed TMA blocks as previously described [16]. Briefly, double tissue cores with a diameter of 2 mm were obtained from each donor block, and these core specimens were transferred to a recipient block using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD, USA). One TMA block contained a maximum of 26 tumor samples, and 13 TMA sets were used in this study.

Immunohistochemistry

Immunohistochemistry was performed on TMA sections of 220 patients. The antibodies used were mouse monoclonal anti-human NS (clone BL2858; Bethyl Laboratories, Inc., Montgomery, TX, USA) and mouse monoclonal anti-human p53 (clone DO-7; Dako, Carpinteria, CA, USA). Formalin-fixed paraffin-embedded specimens on the TMA were cut into 4 μm-thick sections. The tissue sections were deparaffinized twice in xylene for 10 min and rehydrated through graded ethanol (99%, 90%, 80%, and 70%) to water. Antigens were retrieved by microwave heating for 30 min in 10 mM sodium citrate (pH 6.0) for NS and by autoclaving for 15 min in 10 mM Tris–HCl (pH 9.0) for p53. To block endogenous peroxidase activity, the sections were treated with 100% methanol containing 3% hydrogen peroxide for 5 min. Non-specific binding was blocked by incubation in 2% normal swine serum (Dako) in phosphate-buffered saline. The slides were incubated with primary antibodies at 4°C overnight and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision Plus; Dako) for 30 min at room temperature. Specific antigen–antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. Counterstaining was performed using Mayer's hematoxylin. A separate assay was run using a case of esophageal carcinoma as a positive control for NS [17]. Reactions without the primary antibodies were used as negative controls.

Table 1 Correlation between nucleostemin expression and clinicopathological variables in surgically resected breast cancers

Variable	Number of cases (%)			
		Nucleostemin expression		
	Total	Positive	Negative	P-value
	(n = 220)	(n = 142)	(n = 78)	
Age				
Median (range)		52 (30 ~ 82 y)		
≦52	109	71 (65)	38	0.89
>52	111	71 (64)	40	
Tumor size				
<5.0 cm	174	108 (62)	66	0.21
≧5.0 cm	42	31 (74)	11	
Unknown	4	4(100)	0	
Lymph node metastasis				
Negative	115	70 (61)	45	0.39
Positive	101	68 (67)	33	
Unknown	4	4 (100)	0	
Distant metastasis				
Negative	209	134 (64)	75	0.72
Positive	8	6 (75)	2	
Unknown	3	2 (67)	1	
Stage				
I or II	179	111 (61)	68	0.13
III or IV	37	28 (76)	9	
Unknown	4	3 (75)	1	
Nuclear grade				
1, 2	137	86 (63)	51	0.48
3	83	56 (67)	27	
ER status				
Negative	88	50 (57)	38	0.050
Positive	132	92 (70)	40	
PgR status				
Negative	96	57 (59)	39	0.16
Positive	124	85 (69)	39	
HR (ER/PgR) status				
Negative	66	40 (61)	26	0.42
Positive	154	102 (66)	52	
HER2 status				
Negative	190	117 (62)	73	0.02
Positive	30	25 (83)	5	
p53 status				
Negative	143	85 (59)	58	0.03
Positive	77	57 (74)	20	
Histological type				
Ductal	191	125 (65)	66	0.38
Lobular	10	5 (50)	5	
Mucinous	6	6 (100)	0	
Tubular	5	1 (20)	4	
Medullary	3	2 (67)	1	
Other	5	3 (60)	2	

Abbreviation: ER Estrogen receptor, *PgR* Progesterone receptor, *HR* Hormone receptor.

NS and p53 expression was assessed according to the proportion of nuclear staining area. Specimens with 10% or more immunoreactive tumor cells were considered positive, and those with less than 10% were considered negative. Immunohistochemistry results were independently evaluated by two observers (T.K. and H.T.), and cases with discrepant grades were re-evaluated by discussion until consensus was achieved.

ER, PgR, and HER2 had already been immunohistochemically re-assessed on new sections in our previous study [16] by using mouse monoclonal anti-human ER (clone 1D5, Dako), mouse anti-human PgR (clone PgR636, DAKO), and rabbit polyclonal anti-HER2 antibody (HercepTest kit, Dako) according to the methods recommended by the manufacturer. ER and PgR were considered positive if the nuclear staining was observed in 10% or more of tumor cells. Samples were considered hormone receptor positive if they were ER and/or PgR positive and hormone receptor negative if they were ER and PgR negative. HER2 results were considered positive if the IHC score was "3+" or gene amplification was detected by FISH according to the 2007 ASCO/CAP guideline [18].

Statistical analysis

Comparisons between groups were evaluated using chi-squared test or Fisher's exact test. Disease-free survival (DFS) curves of patients were drawn using the Kaplan-Meier method and compared using the log-rank test. Cox multivariate proportional hazards models were used to explore the association of variables with DFS. For all tests, $P < 0.05$ was considered to be statistically significant. All analyses were performed using the software JMP 6.0 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Clinicopathological and prognostic implications of NS expression for the entire patient cohort

Initially, the expression levels of NS were classified as negative (0%), weak (1% to <10%), moderate (10% to <30%), or strong (30% or more). The number of cases categorized

into the negative, weak, moderate, and strong groups was 62, 16, 55, and 87, respectively. From these results, we judged that NS expression showed bimodal distribution and used a 10% threshold for NS positivity between negative and positive groups.

NS protein was frequently detected in the nucleus of breast cancer cells. Although strong immunoreaction was observed in both the nucleoli and nucleoplasm of cancer cells (Figure 1A), nuclear immunoreaction of NS in some cases was limited to the nucleoli of cancer cells (Figure 1B). Such cells were also judged as positive for NS immunoreactivity. Cytoplasmic staining was not observed. These findings are consistent with those of previous reports [6,17,19]. We found that 78 (35.5%) or 142 (64.5%) patients had NS-negative or NS-positive tumors, respectively (Figure 1C). Unremarkable mammary glands showed nuclear NS immunoreactivity in almost all luminal epithelial cells (Figure 1D).

Tumors with NS positivity showed a higher frequency of ER positivity, HER2 positivity, and p53 positivity ($P = 0.050$, $P = 0.021$, and $P = 0.031$, respectively), whereas NS expression status was not correlated with tumor size, lymph node metastasis, distant metastasis, tumor nuclear grade, or PgR positivity. NS expression was detected at 50% or more in all histological types studied except tubular carcinoma (20%), and the positive rate was 100% (6 of 6) in mucinous carcinoma. Patients with NS-positive tumors showed significantly shorter DFS time than those with NS-negative tumors ($P = 0.020$, Figure 2).

Prognostic implication of the NS and p53 combination status for the entire patient cohort

Since it has been reported that physical and functional interaction between NS and p53 appear to be essential for self-renewal, cell cycle regulation, cell proliferation, and apoptosis [7], we next examined the prognostic implication of the combination status of NS and p53 for the entire patient cohort. We found that 143 (65%) and 77 (35%) patients had p53-negative and p53-positive tumors, respectively. The patients with p53-positive tumors showed significantly shorter DFS time than those with p53-negative tumors ($P = 0.006$, Figure 3A).

A striking stratification of relapse risk was identified when three different combinations of NS and p53 status were evaluated: 57 cases with a combination of NS-positive/p53-positive tumors (unfavorable group); 105 cases comprising 20 NS-negative/p53-positive tumors and 85 NS-positive/p53-negative tumors (intermediate group); and 58 cases with NS-negative/p53-negative tumors (favorable group). The unfavorable group had a 5-year DFS rate of 55%, compared with 75% in the intermediate group and 86% in the favorable group (Figure 3B). The unfavorable group had significantly shorter DFS time than the intermediate and favorable groups (log-rank test $P = 0.034$ and $P = 0.0007$, respectively).

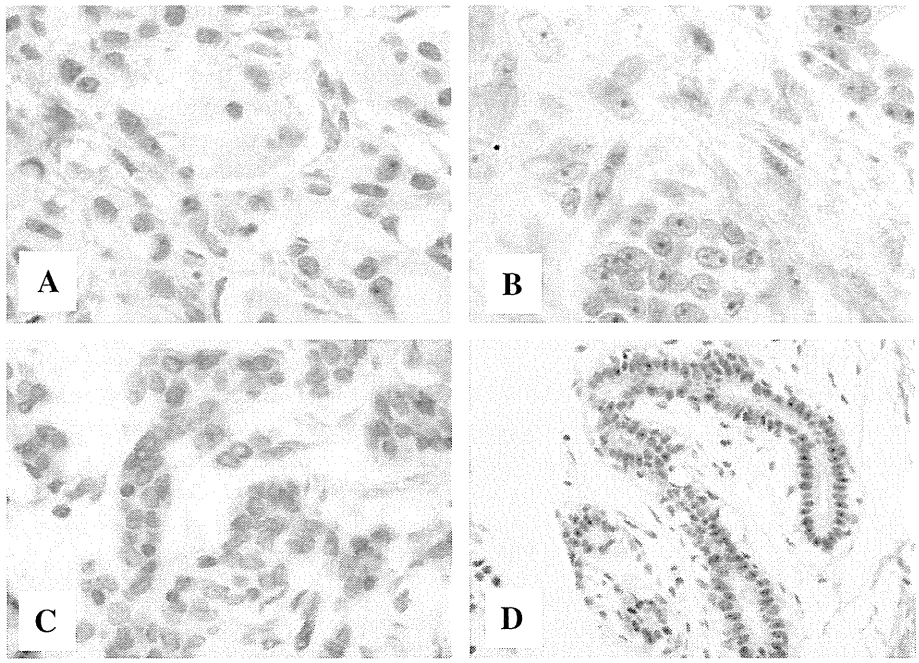

Figure 1 Nucleostemin (NS) expression in human breast cancer tissues. A. A NS-positive tumor. Almost all cancer cells show NS immunoreactivity in both nucleoli and nucleoplasm. **B.** Another NS-positive tumor, NS immunoreactivity is limited to nucleoli in nuclei of cancer cells. Such cancer cells are also judged as NS-positive. This case was also classified as NS-positive. **C.** A NS-negative tumor. **D.** An unremarkable mammary gland shows nuclear NS immunoreactivity in almost all luminal epithelial cells.

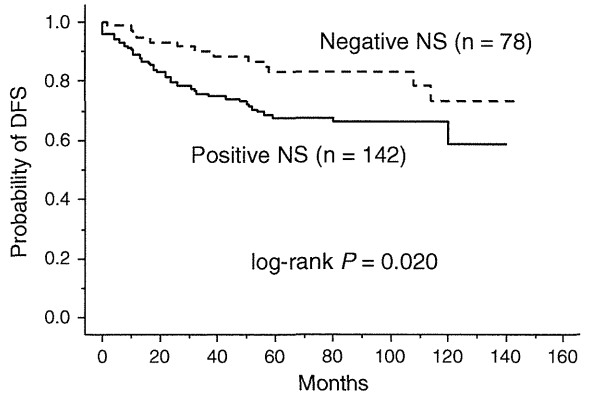

Figure 2 Prognostic impact of NS status in the patients with primary breast cancer. This figure shows disease-free survival (DFS) curves for the 142 patients with NS-positive tumors and for the 78 patients with NS-negative tumors. These two curves differ significantly ($P = 0.020$).

Prognostic implication of NS among the three biological subtypes of breast tumors

Currently, treatment strategies differ between the biological subtypes of breast tumors; therefore, we examined the prognostic implication of NS among three groups of patients divided based on their biological subtype: 154 patients with luminal-type tumors (HR-positive); 22 patients with HER2-type tumors (HER2-positive and HR-negative); and 44 patients with triple-negative tumors (HR and HER2-negative). Eight patients with HR-positive and HER2-positive tumors were included and analyzed as luminal-type patients.

Among the patients with luminal-type tumors, patients with NS-positive tumors showed a significantly shorter DFS time than those with NS-negative tumors ($P = 0.033$, Figure 4A). Among the patients with HER2-positive tumors, patients with NS-positive tumors had a 5-year DFS rate of 28% compared with 100% in patients with NS-negative tumors (Figure 4B). However, the *P*-value was not calculated because there was no relapse

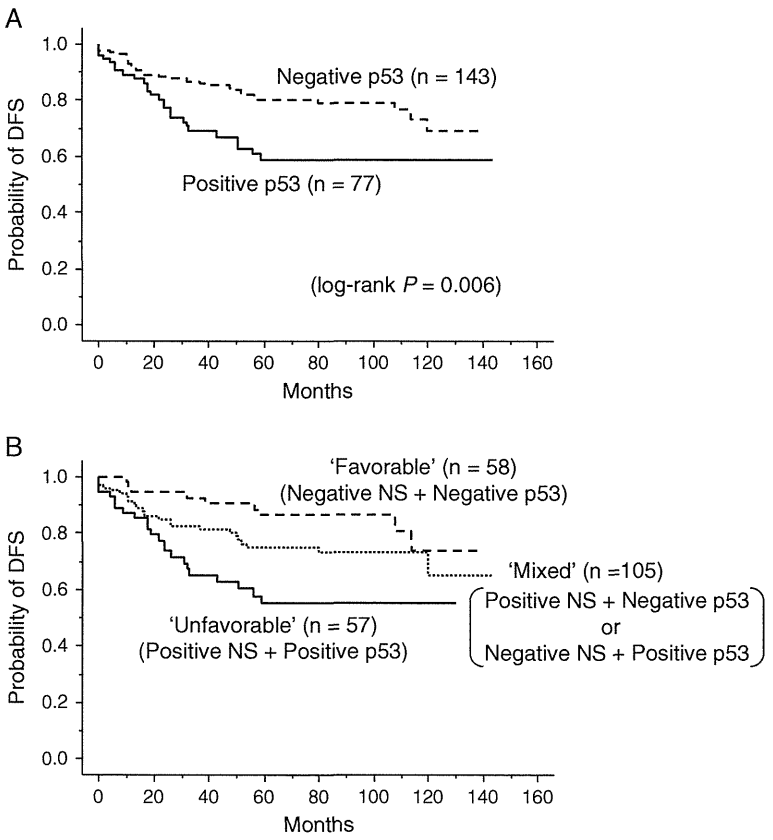

Figure 3 Prognostic impact of the combination status of NS and p53 in the patients with primary breast cancer. A. Disease-free survival (DFS) curves for the 77 patients with p53-positive tumors and for the 143 patients with p53-negative tumors. These two curves differ significantly ($P = 0.006$). **B.** This figure shows three disease-free survival (DFS) curves: for the 57 patients with NS-positive and p53-positive tumors ('unfavorable group'), for 105 patients comprising 20 NS-negative and p53-positive tumors and 85 NS-positive and p53-negative tumors ('intermediate' group), and for 58 patients with NS-negative and p53-negative tumors ('favorable' group). The unfavorable group has significantly shorter DFS time than the intermediate group or the favorable group (log-rank test $P = 0.034$ and $P = 0.0007$, respectively).

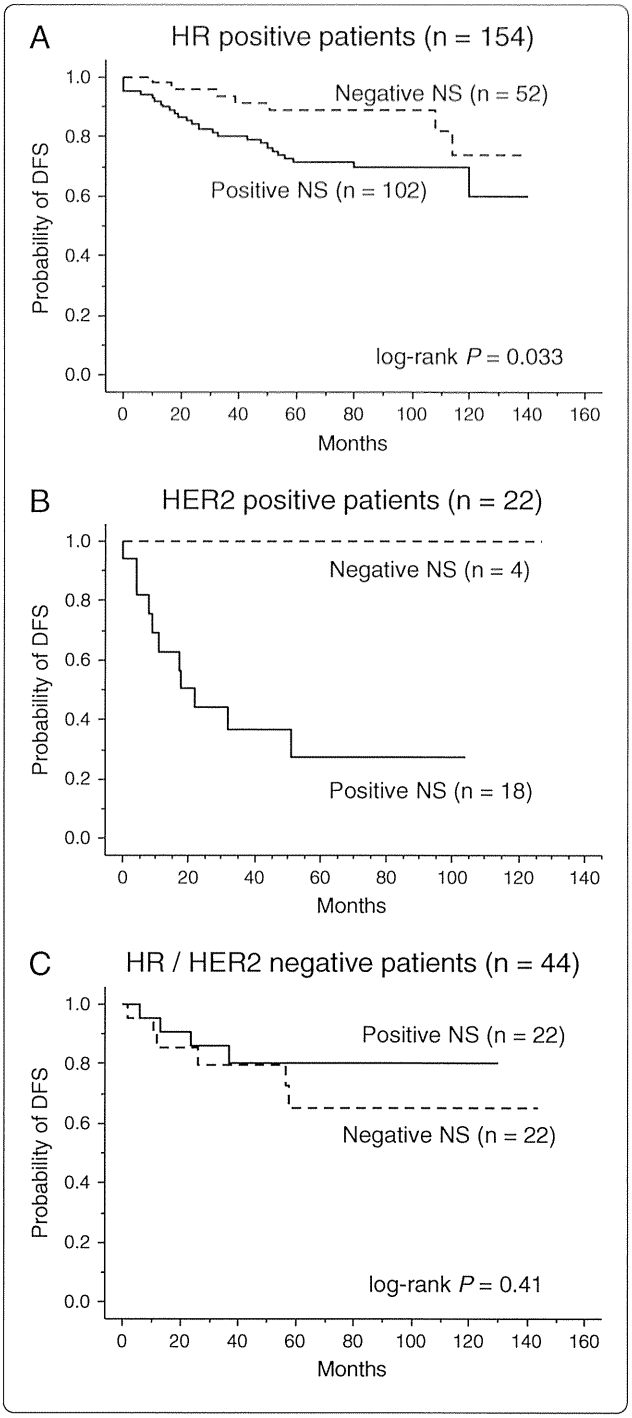

Figure 4 Prognostic impact of NS status in the three subgroups of the different biological subtype tumors: luminal-type tumors, HER2-type tumors, and triple-negative tumors. **A**. Subgroup analysis of the 154 patients with luminal-type tumors (HR-positive tumors). Two disease-free survival (DFS) curves, that for the 102 patients with NS-positive tumors and that for the 52 patients with NS negative tumors, differ significantly ($P = 0.033$). **B**. Subgroup analysis of the 22 patients with HER2-type tumors (HER2-positive and HR-negative tumors). In two disease-free survival (DFS) curves, that for the 18 patients with NS-positive tumors and that for the 4 patients with NS-negative tumors, five-year DFS rates differ largely (100% vs 28%). *P*-value was not available because there was no relapse in the patients with NS-negative tumors. **C**. Subgroup analysis of the 44 patients with triple-negative tumors (HR and HER2 negative tumors). The two curves do not differ significantly ($P = 0.41$).

in the four patients with NS-negative tumors. Among the patients with triple-negative tumors, there was no difference between the survival curves for patients with NS-positive tumors and those with NS-negative tumors ($P = 0.41$, Figure 4C).

Multivariate analysis of prognostic factors and evaluation of NS

Univariate analysis showed that HR, HER2, nuclear grade, tumor size, nodal status, distant metastasis, and NS expression were significantly correlated with DFS. When multivariate analysis was performed using these seven factors, NS expression status was selected as an independent prognostic factor ($P = 0.036$), together with nuclear grade, tumor size, lymph node status, and distant metastatic status ($P = 0.0008$, 0.0007, 0.0038 and <0.0001, respectively; Table 2).

Discussion

In the present cohort, we found that the NS protein expression status was positively correlated with both ER and HER2 status and was a powerful prognostic factor. Patients with NS-positive breast tumors had a significantly shorter DFS time than those with NS-negative tumors ($P = 0.020$, Figure 2), and multivariate analysis for DFS showed that NS positivity had an independent impact as a prognostic indicator among breast cancer patients ($P = 0.036$, Table 2). To our knowledge, this is the first report to show the clinical implication of NS protein expression in invasive breast cancers.

Although several studies have shown the important roles of NS in the pathogenesis of various cancer types [8-13] as well as the maintenance of cancer stem cells [14,15], no direct evidence is yet available to support that NS is a marker of cancer stem cells. Currently, molecules such as CD44, CD133, ALDH1, and CXCR4 have been found to be potential markers of cancer stem cells [20-25]. Furthermore, the expression of these stem cell markers has been shown to be a poor prognostic