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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Verification of reverse-phase plasma microarray (RPPM).

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Phase II study of erlotinib plus gemcitabine in Japanese patients with unresectable pancreatic cancer

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Erlotinib combined with gemcitabine has not been evaluated in Japanese patients with unresectable pancreatic cancer. This two-step phase II study assessed the safety and pharmacokinetics of erlotinib 100 mg/day (oral) plus gemcitabine 1000 mg/m^2 (i.v. days 1, 8, 15) in a 28-day cycle in the first step, and efficacy and safety in the second step. The primary end-point was safety. One hundred and seven patients were enrolled (first step, $n = 6$; second step, $n = 101$). The most common adverse event was RASH (compiled using the preferred terms rash, acne, exfoliative rash, dermatitis acneiform, erythema, eczema, dermatitis and pustular rash) in 93.4% of patients. One treatment-related death occurred. While interstitial lung disease-like events were reported in nine patients (8.5%; grade 1/2/3, 3.8/2.8/1.9%), all patients recovered or improved. The median overall survival, the 1-year survival rate and median progression-free survival were 9.23 months, 33.0% and 3.48 months, respectively. The overall response and disease control rates were 20.3% and 50.0%, respectively. In Japanese patients with unresectable pancreatic cancer, erlotinib plus gemcitabine had acceptable toxicity and efficacy that was not inferior to that seen in Western patients. (*Cancer Sci* 2011; 102: 425–431)

Approximately 232 000 individuals are diagnosed with pancreatic cancer worldwide each year, with an annual death rate estimated at 227 000.[1] In Japan, approximately 22 000 new cases were reported in 2005.[2] Furthermore, data from 2007 show that around 24 000 individuals in Japan died from pancreatic cancer, making this tumor type the fifth leading cause of cancer-related death.[3] The majority of pancreatic cancer cases are diagnosed at an unresectable stage when prognosis is extremely poor.

Current treatment for advanced pancreatic cancer is based on systemic chemotherapy with gemcitabine. Single-agent gemcitabine has been shown to extend median overall survival (OS) to 5.65 months in chemonaïve patients compared with 4.41 months in patients who received fluorouracil.[4] Addition of other cytotoxic agents to gemcitabine has not demonstrated survival benefits over gemcitabine alone.[5–13] The potential of combining gemcitabine with biological agents in patients with advanced pancreatic cancer has also been evaluated in several phase III studies, but these trials failed to show a survival benefit.[14–19]

Epidermal growth factor receptor (EGFR)-mediated signaling is associated with various cellular processes, and the dysregulation of these processes is common in tumorigenesis.[20,21] Furthermore, EGFR is overexpressed in many tumors and its overexpression is often associated with poor prognosis.[22–26] EGFR tyrosine-kinase inhibitors (TKI, such as erlotinib) are used in the treatment of various types of solid tumors.

Erlotinib has demonstrated antitumor activity in pancreatic cell lines[27] and was subsequently assessed as a potential therapeutic agent in pancreatic cancer. In the PA.3 study ($n = 569$), the risk of death with erlotinib plus gemcitabine was reduced by 18% versus gemcitabine alone (hazard ratio [HR], 0.82; 95% confidence interval [CI], 0.69–0.99; $P = 0.038$ after adjustment for stratification factors), with a median OS of 6.24 months *vs* 5.91 months, respectively. Erlotinib plus gemcitabine combination therapy provided significant improvements in the 1-year survival rate (23% *vs* 17%; $P = 0.023$) and progression-free survival (PFS; HR 0.77; 95% CI, 0.64–0.92; $P = 0.004$).[28] As a result, this combination was approved for use in pancreatic cancer in many countries.

In Japanese patients with non-small-cell lung cancer (NSCLC), a phase II study has specifically shown that erlotinib monotherapy is well tolerated and has promising antitumor activity.[29] However, there are no data on the use of erlotinib combined with gemcitabine in Japanese patients with pancreatic cancer. This phase II study evaluated the safety and efficacy of erlotinib in combination with gemcitabine in Japanese patients with unresectable locally advanced or metastatic pancreatic cancer.

Methods

Patients. Patients aged 20–80 years with histological/cytological evidence of unresectable locally advanced or metastatic adenocarcinoma/adenosquamous carcinoma of the pancreas were eligible for inclusion in the present study. Patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2, adequate hematological, renal and hepatic function and a life expectancy of at least 2 months. No more than one prior regimen for pancreatic cancer was permitted. Patients who had received prior gemcitabine and/or a TKI were excluded from participation, as were those who had previously been exposed to a human epidermal growth factor receptor 2 (HER2) or EGFR inhibitor. Other key

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Clinical trial registry: JAPIC Clinical Trials Information (see links below). http://rctportal.niph.go.jp/examDetail.php?center=3&center_seq=698 http://www.clinicaltrials.jp/user/cteDetail.jsp?clinicalTrialId=839&language=ja. Trial registration number: JapicCTI-060337.

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exclusion criteria were: symptomatic cerebral metastases; a concurrent lung disorder (such as idiopathic pulmonary fibrosis, interstitial lung disease [ILD] or pneumoconiosis); concurrent or previous drug-induced pneumonia; or a history of radiation to the chest.

The study complied with the Declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was obtained from all patients, and the protocol was approved by ethics committees at all participating institutions.

Study design and treatment. This was a phase II, multicentre, open-label, two-step study. In the first step, six patients were enrolled into the study and treated with oral erlotinib 100 mg/day on days 3–28, plus i.v. gemcitabine 1000 mg/m^2 on days 1, 8 and 15 in a 28-day cycle. The starting doses of erlotinib and gemcitabine were chosen in reference to the PA.3 study. Dose-limiting toxicities (DLT) were assessed in these study participants using the National Cancer Institute Common Terminology Criteria for Adverse Events v3.0 (NCI-CTCAE, National Cancer Institute, Bethesda, MD, USA). Dose-limiting toxicities were defined in conformity to the P1b study as follows:[30] (i) grade 4 decrease (i.e. to <500/mm^3) in neutrophil count >5 days; (ii) grade ≥3 decrease (i.e. to <1000/mm^3) in neutrophil count with associated fever (≥38.5°C); (iii) grade 4 decrease in platelet count (i.e. to <25 000/mm^3); (iv) any grade ILD; (v) grade 4 elevation of alanine transaminase (ALT)/aspartate transaminase (AST) levels, or grade 3 elevation of ALT/AST levels >7 days; (vi) grade ≥3 non-hematological toxicity (excluding rash, hyperglycemia, γ-GTP and events that were judged to be transient/had no effect on study continuation); and (vii) dose-reduction/interruption required due to persistent adverse events (AE), which meant that the second cycle could not be started.

If treatment-related DLT occurred in no more than two of the six patients, transition to the second step of the study was permissible with approval of the Data Safety and Monitoring Committee (DSMC). If DLT occurred in three or more patients, transition to the second step was limited to those cases that were judged to be safe for this study after the DSMC had evaluated the safety data of the patients with a DLT. In the second step, it was planned that 94 patients would be treated with the same dose as the first step. Treatment was continued until disease progression, death, unacceptable toxicity or patient/investigator request.

The primary end-point of the study was safety, with secondary end-points including OS, 1-year survival rate, PFS, overall response rate (ORR), disease control rate (DCR = complete response [CR] + partial response [PR] + stable disease), pharmacokinetics (PK) and correlation of *EGFR* mutation status with outcomes.

Toxicity evaluation. Adverse events were monitored and graded using NCI-CTCAE v3.0. Clinical and laboratory assessments were conducted throughout the study. Adverse events prespecified in the study to be monitored carefully were rash, diarrhea, vomiting, liver dysfunction and ILD-like events. Chest X-ray examination to assess pulmonary toxicity was conducted weekly until week 4 and every 2 weeks thereafter. In addition, chest computed tomography (CT) scan was performed every 4 weeks. The DSMC reviewed the images and clinical data associated with all potential ILD-like events. All ILD-like events were reported to be serious AE (SAE), regardless of the grade.

Efficacy evaluation. The tumor response was assessed using Response Evaluation Criteria in Solid Tumors (RECIST) in patients who had at least one measurable target lesion. Tumors were measured using computed tomography (CT) at baseline and on day 22 of every two cycles thereafter. Median PFS, ORR and DCR were estimated by the extramural review. The relationship between efficacy and the severity of RASH (compiled using the preferred terms rash, acne, exfoliative rash, dermatitis acneiform, erythema, eczema, dermatitis and pustular rash) was also examined.

Pharmacokinetic evaluation. Pharmacokinetic evaluation of erlotinib and its O-desmethylated metabolite (OSI-420) was performed in the six patients enrolled in the first step of the study. Venous blood samples were taken prior to erlotinib dosing on day 3 and day 8 of cycle 1 at 0.5, 1, 2, 4, 6, 8 and 24 h after erlotinib administration. Samples were also taken prior to gemcitabine infusion on days 1 and 8 at 0.5, 0.75, 1, 1.5, 2.5 and 4.5 h after dosing.

The plasma concentrations of erlotinib, OSI-420 and gemcitabine were measured by liquid chromatography, tandem mass spectrometry (LC-MS-MS). The LC-MS-MS analytical methods have been described previously.[31,32] Derived PK parameters included the maximum plasma drug concentration (C_{max}), time to C_{max} (t_{max}), area under the plasma drug concentration-time curve to the last plasma sample (AUC_{last}), terminal half-life ($t_{½}$) and oral clearance (Cl/F).

Biomarker analysis. *EGFR* mutations were assessed in patients with available tumor tissue specimens, which were formalin fixed and paraffin embedded. Samples were analyzed at a central laboratory where DNA was extracted and exons 18–21 sequenced using a nested PCR.

Statistical analysis. Progression-free survival and OS were estimated using the Kaplan–Meier method in all patients who received at least one dose of the study treatment, with 95% CI for the median duration calculated using Greenwood's formula. The Clopper–Pearson method was used to calculate the 95% CI around the ORR, DCR and AE rate. Multivariate analyses were performed for the occurrence of ILD-like events using the logistic regression model. Baseline characteristics investigated for this analysis included gender, age, lung metastasis, emphysema and various baseline laboratory values. The target enrollment was 100 patients, as this was required to evaluate the safety of erlotinib.

Results

Patient characteristics. Between December 2006 and October 2007, a total of 107 patients were enrolled (first step, $n = 6$; second step, $n = 101$) from 12 institutions (Fig. 1). One patient who enrolled into the second step did not receive treatment due to deterioration in PS prior to the start of treatment. A total of 106 patients were evaluable for safety (safety population, full analysis set).

The patient demographics and baseline characteristics are shown in Table 1. The median age was 62 years (range, 36–78) and 52.8% of patients were male. Almost all patients were chemonaïve (95.3%). The majority (75.5%) of patients had an ECOG PS of 0 and most (83.0%) had metastatic disease. Over half (63.2%) of the patients had a history of current or past smoking.

Toxicity and dose modifications. The median duration of erlotinib exposure was 102.5 days and its median dose intensity was 100.0 mg/day, with the majority of patients (78.3%) receiving more than 90% of the relative dose intensity. The median duration of gemcitabine treatment was 4.0 cycles and its median dose intensity was 688.0 mg/m^2 per week, with approximately half of the patients (51.4%) receiving more than 90% of the relative dose intensity.

As only one patient had a DLT (grade 3 diarrhea) in the first step, the second step of the study was initiated. One hundred and six patients received at least one dose of erlotinib; these patients were assessable for toxicity. Treatment-related AE and treatment-related changes in laboratory values are summarized in Table 2; most of these were mild to moderate in severity. The most frequently reported AE was RASH, which occurred in

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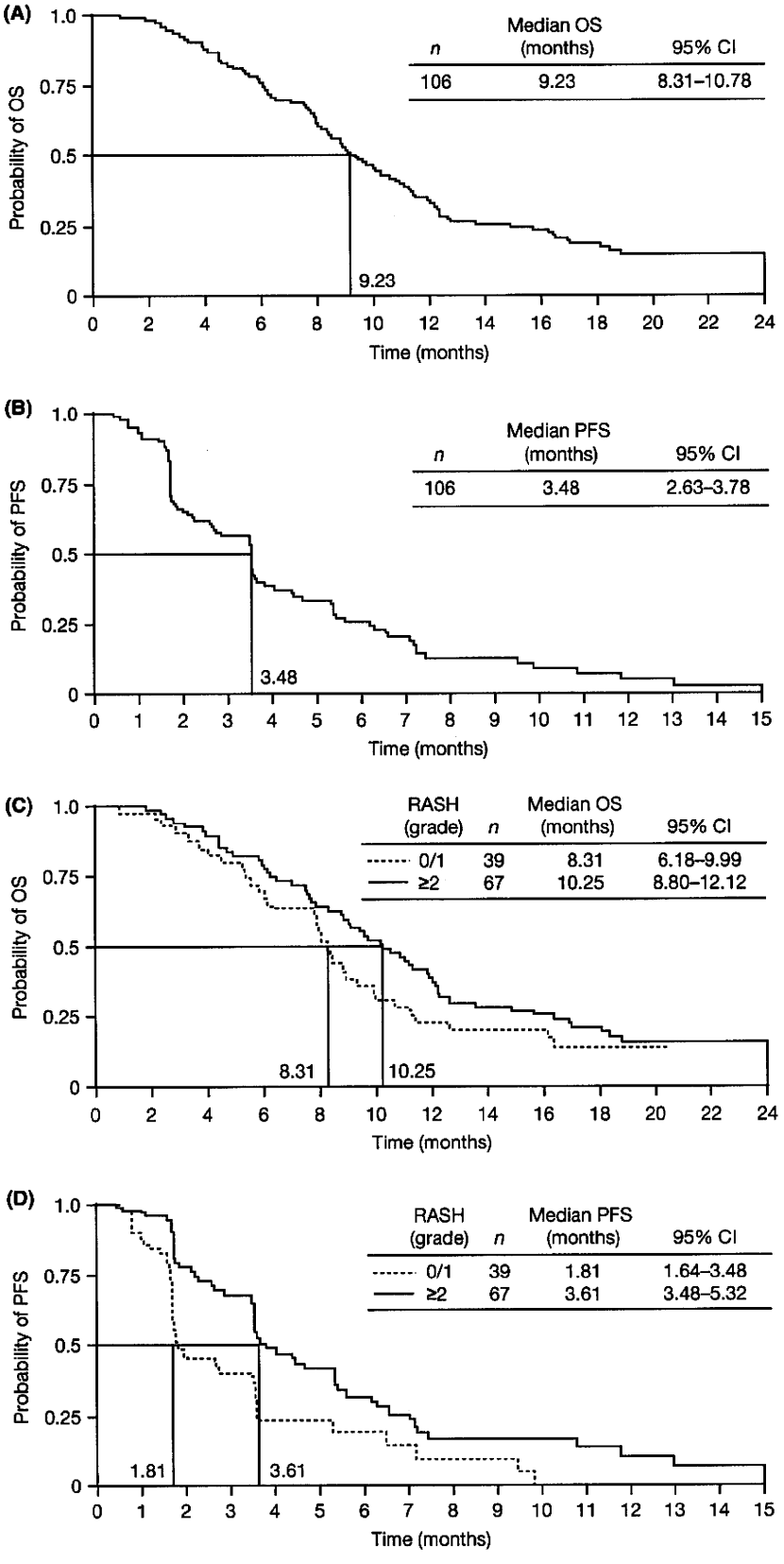

Fig. 1. Kaplan–Meier estimates of (A) overall survival (OS) and (B) progression-free survival (PFS) in the study population (n = 106); (C) OS and (D) PFS according to the severity of RASH (grade ≤1 [n = 39] *vs* grade ≥2 [n = 67]). RASH is a composite of the terms: rash, acne, exfoliative rash, dermatitis acneiform, erythema, eczema, dermatitis and pustular rash. CI, confidence interval.

Table 1. Baseline characteristics and demographics (n = 106)

Characteristic	
Median age (range) (years)	62 (36–78)
Gender, n (%)	
Male	56 (52.8)
Female	50 (47.2)
Median bodyweight (range) (kg)	52.3 (33.1–95.0)
Smoking history,† n (%)	
Never smoker	39 (36.8)
Past smoker	37 (34.9)
Current smoker	30 (28.3)
ECOG PS, n (%)	
0	80 (75.5)
1	26 (24.5)
2	0 (0)
Disease status, n (%)	
Metastatic	88 (83.0)
Locally advanced	18 (17.0)
Primary tumor identified, n (%)	92 (86.8)
Primary sites, n (%)	
Head	46 (43.4)
Body and tail	23 (21.7)
Body	22 (20.8)
Tail	10 (9.4)
Other	5 (4.7)‡
Biliary drainage, n (%)	19 (17.9)
Sites of distant metastases, n (%)	
Liver	56 (52.8)
Distant lymph nodes	39 (36.8)
Lung	17 (16.0)
Other	26 (24.5)
Prior lines of therapy, n (%)	
None	101 (95.3)
One regimen	5 (4.7)§
Median CA19–9 (range) (U/mL)	
Median	776 (0–435 000)
Median CEA (range) (ng/mL)	
Median	4.8 (0.6–1100.1)

†Never smoker, never/hardly smoked; past smoker, passage of at least 1 month since stopping smoking (at the time of registration); current smoker, smoked within 1 month (at the time of registration). ‡Whole of pancreas (n = 1); head and body (n = 3); other (n = 1). §Tegafur, gimeracil, oteracil potassium (S-1) (n = 3); 5-fluorouracil plus leucovorin (n = 2). CA 19–9, carbohydrate antigen 19–9; CEA, carcinoembryonic antigen; ECOG, Eastern Co-Operative Group.

93.4% of the patients; most cases were mild to moderate in severity (87.7%, grade ≤2; 5.7%, grade ≥3). Other common non-hematological AE included anorexia, pruritus, fatigue, nausea and diarrhea. Most patients experienced some degree of hematological toxicity, with grade 3 or 4 neutropenia (neutrophil decreased), leucopenia (white blood cell count decreased) and anemia (hemoglobin decreased) occurring in 34.9%, 29.2% and 14.2% of patients, respectively. Only one treatment-related death occurred (due to gastrointestinal hemorrhage), which was probably due to arterial bleeding caused by the invasion of the primary tumor into the gastrointestinal tract. Although the likelihood of this event being treatment-related was deemed remote, a causal relationship could not be completely excluded because the event occurred during the study treatment administration period.

Treatment-related SAE were reported in 26 (24.5%) patients. These included nine ILD-like events (8.5%), the majority of which (n = 7) were grade 1–2 in severity. Importantly, all of these nine patients recovered or improved, and four of these patients did so without any treatment for ILD-like events. Other

Table 2. Treatment-related adverse events occurring in >30% of patients treated with erlotinib and gemcitabine (n = 106)

	Any grade, n (%)	Grade 3, n (%)	Grade 4, n (%)
Non-hematological			
Rash	78 (73.6)	3 (2.8)	0 (0)
Anorexia	75 (70.8)	15 (14.2)	0 (0)
Pruritus	57 (53.8)	1 (0.9)	0 (0)
Fatigue	56 (52.8)	3 (2.8)	0 (0)
Nausea	56 (52.8)	6 (5.7)	0 (0)
Diarrhea	52 (49.1)	2 (1.9)	0 (0)
Dry skin	49 (46.2)	0 (0)	0 (0)
Stomatitis	38 (35.8)	0 (0)	0 (0)
Pyrexia	32 (30.2)	0 (0)	0 (0)
Hematological			
White blood cell count decreased	85 (80.2)	31 (29.2)	0 (0)
Platelet count decreased	77 (72.6)	9 (8.5)	0 (0)
Hemoglobin decreased	76 (71.7)	13 (12.3)	2 (1.9)
Hematocrit decreased	73 (68.9)	8 (7.5)	0 (0)
Neutrophil decreased	73 (68.9)	32 (30.2)	5 (4.7)
Red blood cell count decreased	72 (67.9)	8 (7.5)	0 (0)
ALT increased	59 (55.7)	10 (9.4)	0 (0)
AST increased	57 (53.8)	4 (3.8)	1 (0.9)
Weight decreased	53 (50.0)	3 (2.8)	0 (0)
Lymphocyte count decreased	46 (43.4)	14 (13.2)	0 (0)
Blood albumin decreased	35 (33.0)	0 (0)	0 (0)
Gamma-glutamyltransferase increased	35 (33.0)	12 (11.3)	1 (0.9)

ALT, alanine amino transferase; AST, aspartate amino transferase.

treatment-related SAE were anorexia (3.8%), vomiting, pyrexia and abnormal hepatic function (1.9% each). The baseline characteristics, treatment and outcomes of patients who developed treatment-related ILD-like events during the study are detailed in Table 3. The onset times of ILD-like events ranged from 7 to 187 days after the start of treatment. In these patients, a relatively long survival was observed (from 119 to 568+ days), and five patients received post-study therapy. All of these nine patients were past or current smokers, and six had emphysema at baseline (not detected prior to treatment, but diagnosed at the extramural review by a radiologist in the DSMC). Multivariate analyses were performed for the occurrence of ILD-like events using the logistic regression model and emphysema at baseline was indicated as a risk factor for onset of ILD-like events (odds ratio [95% CI], 12.13 [1.01–145.7]; P = 0.0491).

Adverse events led to erlotinib discontinuation in 30 patients (28.3%) and gemcitabine discontinuation in 27 patients (25.5%). The main reasons for treatment discontinuation were ILD (n = 6) and anorexia (n = 3); no patient discontinued treatment due to RASH or diarrhea. Due to the onset of AE, a total of 65 patients (61.3%) required one or more interruptions of erlotinib (36 patients [34.0%] for longer than seven consecutive days and 17 patients [16.0%] for longer than 14 consecutive days) and 56 patients (52.8%) had one or more skip of gemcitabine. Modifications in the erlotinib or gemcitabine dosage were required in 17 (16.0%) and 11 (10.4%) patients, respectively, due to AE.

Efficacy. The median OS was 9.23 months (95% CI, 8.31–10.78; Fig. 1A) and the 1-year survival rate was 33% (95% CI, 24–42). Median PFS was 3.48 months (95% CI, 2.63–3.78; Fig. 1B). Among the patients evaluable for tumor response (n = 64), the ORR was 20.3% (13/64; 95% CI, 11.3–32.2) and the DCR was 50.0% (95% CI, 37.2–62.8; CR, n = 0; PR, n = 13; stable disease, n = 19).

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Table 3. Characteristics, treatment and outcomes of patients with treatment-related ILD-like events (n = 9)

Event	Gender	Age (years)	Smoking status†	Days on treatment	ILD maximum grade	Suspicious findings of ILD	Steroids	Oxygen	ILD outcome	Presence of emphysema (assessed by radiologist)	Survival outcome (days)	Post-therapy (chemotherapy)
Lymphoid ILD	M	62	Past	82	1	Pyrexia	None	No	Improved	Yes	362	Yes
ILD	M	42	Current	50	3	Pyrexia	Pulse	Yes	Recovered	Yes	517	Yes
Organising pneumonia	M	60	Past	183	2	Respiratory symptoms	None	No	Improved	Yes	568+	Yes
ILD	F	62	Past	113	2	Cough	Oral	No	Recovered	Yes	376	No
ILD	F	74	Past	111	3	Cough, dyspnea	Pulse	Yes	Improved	None	183	No
ILD	M	60	Current	25	1	Pyrexia	Pulse	No	Recovered	None	119	Yes
ILD	M	77	Past	7	1	X-ray	None	No	Recovered	Yes	255	No
ILD	M	55	Past	187	1	CT	None	No	Recovered	Yes	415	No
ILD	F	60	Current	76	2	Cough	Oral	No	Recovered	None	346	Yes

†Past smoker, passage of at least 1 month since stopping smoking (at the time of registration); current smoker, smoked within 1 month (at the time of registration). CT, computed tomography; F, female; ILD, interstitial lung disease; M, male.

The median OS was longer in patients who experienced RASH of grade ≥2 (n = 67) than in those with RASH of grade ≤1 (n = 39) (10.25 months [95% CI, 8.80–12.12] *vs* 8.31 months [95% CI, 6.18–9.99], respectively; Fig. 1C) and the 1-year survival rate was higher (39% [95% CI, 27–50] *vs* 23% [95% CI, 10–36], respectively). Similarly, the median PFS was longer in patients with RASH of grade ≥2 versus those with RASH grade ≤1 (3.61 months [95% CI, 3.48–5.32] *vs* 1.81 months [95% CI, 1.64–3.48]; Fig. 1D). While there was no notable difference in ORR between patients with RASH grade ≥2 and those with grade ≤1 (21.1% [95% CI, 9.6–37.3] *vs* 19.2% [95% CI, 6.6–39.4]), the DCR was higher in those with more severe RASH (60.5% [95% CI, 43.4–76.0] *vs* 34.6% [95% CI, 17.2–55.7]).

Pharmacokinetics. Plasma sampling for PK analyses was performed in all six patients enrolled in the first step. On day 8, the values of C_{max} were 1760 ± 456.9 ng/mL (mean ± SD) for erlotinib, 169.7 ± 64.5 ng/mL for OSI-420 and 22 700 ± 3272.9 ng/mL for gemcitabine. The AUC_{last} was 29 001 ± 6560 h ng/mL, 2748 ± 788 h ng/mL and 10 717 ± 1458 h ng/mL (mean ± SD), respectively. The mean t_{max} was 8.0 h (range, 2.0–23.9 h), 9.0 h (2.0–23.9 h) and 0.51 h (0.45–0.57 h), respectively. Also on day 8, the mean plasma $t_{1/2}$ was 54.92 h (range, 9.25–144.61 h), 32.79 h (10.36–60.46 h), and 0.63 h (0.31–1.14 h), respectively. The Cl/F of erlotinib and gemcitabine showed interindividual variability; the Cl/F on day 8 was 3972.6 ± 772.1 mL/h (mean ± SD; coefficient of variation 19.4%) and 146 580.4 ± 31 101.3 mL/h (21.2%), respectively.

Biomarker analysis. Of the 106 patients enrolled, *EGFR* mutation status was evaluated in 47 patients (44.3%), all of whom had wild-type *EGFR*. The mutation status of the remaining patients was classified as unknown because samples were not available (30.2%), not examined (9.4%) or the results following sequencing were inconclusive (16.0%).

Discussion

This study was designed to initially assess the safety of erlotinib with gemcitabine for Japanese patients with pancreatic cancer, in whom there had been no prior exposure to either drug. As no significant safety concerns were raised in the first step of the study, enrollment of a further 101 patients was performed. Although the incidence of AE in this study was higher than in the PA.3 study, the incidence of grade 3–4 AE was similar.[28] Despite these results, no new AE specific to Japanese patients were observed. As expected, RASH and gastrointestinal events were among the most common AE in this study, and most of these cases were mild to moderate in severity.

Interstitial lung disease-like events were reported in nine patients (8.5%; grade 1/2/3, 3.8/2.8/1.9%) in the current study, while its incidence was reported to be 2.4% in patients treated in the erlotinib plus gemcitabine arm of the PA.3 study.[28] In addition, in Japanese patients with advanced pancreatic cancer, ILD-like events were reported in two (6.1%) of 33 patients treated with gemcitabine plus S-1, and were reported in three (1.1%) of 264 patients with gemcitabine monotherapy, respectively.[33,34] Likewise, the higher incidence of ILD-like events were documented using S-1 or erlotinib in combination with gemcitabine compared with gemcitabine as monotherapy in patients with pancreatic and biliary tract cancer.[35] On another front, outside of Japan, a high incidence of ILD-like events was reported in gemcitabine and paclitaxel combination therapy in patients with NSCLC.[36] From the above information, considering the higher incidence of ILD when gemcitabine is used in combination, an additive effect from such combinations cannot be ruled out.

In NSCLC, Japanese patients have an increased risk of developing ILD-like events when treated with EGFR TKI.[29,37–39] Fatal cases of ILD-like events have been reported following EGFR TKI administration for the treatment of NSCLC.[37–41] Importantly, however, no patients died due to an ILD-like event in this study. Seven patients experienced ILD-like events of grade 1–2 in severity. This may be due to active management of ILD-like cases during the study period. This management included regular and immediate chest X-rays, in addition to diagnosis with CT scans after any early signs and symptoms were observed (e.g. pyrexia, cough or dyspnea), timely discontinuation of the antitumor drugs (as a precautionary measure in case these drugs were associated with the symptoms) and appropriate treatment for the events (including oral/pulse steroids). By appropriately treating the early symptoms of ILD-like events, patients could restart antitumor therapy (chemotherapy: treatment change). In this study, the onset time for ILD-like events varied markedly between patients (7–187 days). It is therefore necessary to monitor the patients throughout the treatment period.

All of the patients who developed ILD in this study were current or past smokers, and smoking status has been shown to be a risk factor for ILD in the NSCLC population.[38] Results from the multivariate analyses in this study suggest that emphysema is also a risk factor for developing ILD; six of the nine

patients with ILD-like events were diagnosed with emphysema at baseline. Although the number of reports of an ILD-like event may have been artificially elevated due to underlying patient baseline characteristics and the active management of ILD-like events, these results demonstrate the need to consider the risk of ILD-like events in Japanese patients treated with TKI. In particular, it is important that chest CT scans are closely checked for the presence of emphysema or comorbid ILD and that pulmonary status is assessed prior to treatment administration.

This study corroborates the results of the combination of gemcitabine and erlotinib shown in the PA.3 study. The median OS in this study of 9.23 months was longer than those reported in trials with gemcitabine alone. In this study, patients who experienced skin toxicity of grade ≥2 had better outcomes than those with less severe toxicity or the overall study population. Retrospective analyses of data from the PA.3 and AViTA studies have found a significant association between the development of skin toxicity and efficacy in patients with pancreatic cancer treated with erlotinib-based therapy, although the precise mechanisms for the association between skin toxicity and effectiveness are unknown.[28,41,42]

Although the presence of mutations in the tyrosine-kinase region of the *EGFR* gene appears to predict a better response to erlotinib in NSCLC,[43,44] this has not yet been evaluated in pancreatic cancer. *EGFR* mutations are very rare in patients with pancreatic cancer;[45–47] indeed in the present study, no *EGFR* mutations were detected. Further work is required to determine whether *EGFR* mutations can be used as predictive markers for improved survival in Japanese patients receiving erlotinib and gemcitabine as treatment for advanced pancreatic cancer.

In conclusion, the present study shows that erlotinib in combination with gemcitabine is generally well tolerated in Japanese patients with advanced pancreatic cancer. This combination is associated with efficacy and survival outcomes, and the results of this study are consistent with the findings of the global PA.3 study.

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ORIGINAL ARTICLE

Predictive factors for the effectiveness of neoadjuvant chemotherapy and prognosis in triple-negative breast cancer patients

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Abstract
Purpose Triple-negative breast cancers (TNBCs) do not derive benefit from molecular-targeted treatments such as endocrine therapy or anti-HER2 therapy because they lack those molecular targets. On the other hand, TNBCs have been shown to respond to neoadjuvant chemotherapy (NAC). In this study, we analyzed TNBC patients who were treated with NAC at Osaka National Hospital over a recent 5-year period to clarify the predictive factors for NAC and prognostic factors.
Patients and methods Thirty-three TNBC patients underwent sequential NAC with anthracycline (FEC100: 5FU 500 mg/m^2, epirubicin 100 mg/m^2, and cyclophosphamide 500 mg/m^2/q3w, 4 courses) and taxanes (paclitaxel 80 mg/m^2/qw, 12 courses or docetaxel 75 mg/m^2/q3w, 4 courses) from May 2003 to July 2008. Pre-therapeutical and surgical specimens were studied for expressions of ER, PgR, HER-2, EGFR, cytokeratin 5/6, Ki-67, p53 and androgen receptor by immunohistochemistry (IHC). We analyzed clinicopathological factors and molecular markers in regard to the response to NAC and prognosis.
Results Pathological complete response (pCR) was achieved in 12 TNBC patients (36%). The pCR rate in the basal-like phenotype was significantly lower than in the non-basal-like phenotype (23 vs. 64%, respectively: $P = 0.02$). High pre-operative expressions of Ki-67 (≥50%) and HER-2 (2+) were considered as predictive factors for a better response from NAC. Pre-operative Ki-67 expression showed a significant correlation with disease-free survival (DFS) and a lower expression of Ki-67 (<50%) after NAC was favorable for DFS among non-pCR patients.
Conclusions A non-basal-like phenotype and higher expressions of Ki-67 and HER-2 (2+) were favorable factors for NAC. However, a higher expression of Ki-67 on the surgical specimen after NAC was also a poor prognostic factor.

Keywords Triple-negative breast cancer · Neoadjuvant chemotherapy · Pathological complete response · Ki-67 · Basal-like phenotype

Abbreviations

TNBC	Triple negative breast cancer
NAC	Neoadjuvant chemotherapy
pCR	Pathological complete response
ER	Estrogen receptor
PgR	Progesterone receptor
AR	Androgen receptor
EGFR	Epidermal growth factor receptor
CK	Cytokeratin

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Introduction

Triple-negative breast cancers (TNBCs) are characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2). These cancers occur in ~20–25% of all breast cancers and are associated with an unfavorable prognosis. They derive no benefit from molecularly targeted treatments such as endocrine therapy or trastuzumab [1]. Therefore, identifying appropriate treatments for TNBC is an important issue.

Recent precise gene expression analysis revealed that TNBC is a heterogeneous group of tumors. One of the subgroups is a basal-like subtype, which is characterized by similar gene expression as the basal/myoepithelial cells of the normal breast [1–5]. Basal-like breast cancer has also been identified with immunohistochemical (IHC) staining of basal markers, such as cytokeratins (CKs) and epithelial growth factor receptor (EGFR). TNBCs without these basal markers are classified as non-basal-like subtypes, which are rare breast cancers, and classifications based on gene expression have not been clarified yet. Non-basal-like tumors are also reported to have a better prognosis than basal-like phenotypes [6, 7]. Because of the lack of targeted therapies and their aggressive clinical behaviors, TNBCs are relevant groups to be investigated for their characteristics. Though TNBCs are considered to have poor prognosis generally, TNBCs have been shown to be chemosensitive.

Neoadjuvant chemotherapy (NAC) in primary breast cancers has been shown to produce an outcome equivalent to that of adjuvant chemotherapy [8, 9]. Patients who show a pathological complete response (pCR) in the primary tumors after NAC have a better prognosis [10]. The pathological responses are important prognostic parameters and can be used as surrogate parameters for clinical outcome, so we analyzed the effects of clinicopathological factors as well as immunohistochemical factors on pathological responses after NAC. However, the paradox that TNBC and HER-2 positive subtypes showed higher chemosensitivity but worse survival due to higher relapse after chemotherapy is also known well [10, 11].

Several biological markers have been proposed as prognostic characteristics in breast cancers. ER, PR and HER-2 are such biological markers as well as being therapeutic markers and Ki-67, p53 and androgen receptor (AR) are shown to be associated with prognosis [12–16]. AR is known to be present in the majority of primary and metastatic invasive breast tumors and is often co-expressed with ER and PR in these tumors. Though little is known about the role of AR in hormonal response, AR expression has been shown to be associated with a better outcome for untreated breast cancer patients [14]. Ki-67 is a nuclear antigen expressed in the G1, S, and G2 phases but not in the G0 or resting phase of the cell cycle. Ki-67 has been established as a proliferation marker in breast cancers and high proliferation activity has been found to have predictive value for the response to NAC [17]. Also p53 expression status has been used as a predictive factor for response to systemic therapy, because tumor cells with non-functional p53 do not respond to systemic therapy due to a failure in apoptosis [13, 15].

Because chemotherapy is the only treatment other than surgery for TNBC, the definition of clinical markers in regard to chemotherapeutic response and prognosis is very important. However, there are still few studies focusing on TNBC. In this study, we analyzed clinicopathological factors, phenotypes, and molecular markers of TNBC in regard to the response to NAC and prognosis.

Patients and methods

Patients and neoadjuvant chemotherapy

One hundred and 63 breast cancer patients underwent NAC with a sequential regimen containing anthracycline (FEC100: 5FU 500 mg/m^2, epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2/q3w, 4 courses) and taxanes (paclitaxel 80 mg/m^2/qw, 12 courses or docetaxel 75 mg/m^2/q3w, 4 courses) at Osaka National Hospital (Osaka, Japan) from May 2003 to July 2008. The criteria for entry were invasive breast cancer patients from 20 to 70 years old with any T and N0-2 disease, who were diagnosed histologically, were absent from distant metastasis and with normal organ functions. Thirty-three patients (20%) among 163 breast cancer patients were identified as TNBCs. The clinical evaluation of the response to NAC was determined by clinical findings, CT and MRI examinations according to RECIST. All patients were included in clinical trials approved by an institutional review board and asked for written informed consent.

Immunohistochemistry

Pre-therapeutical specimens were obtained by the 14G-needle biopsy in all cases and pathological examinations using standard hematoxylin and eosin staining were carried out. Immunohistochemical evaluation for ER, PgR, HER-2, EGFR, CK5/6, Ki-67, p53 and AR in tissue sections were detected using antibodies (ER:Cat.No. 760-2596I, PgR: 760-2816, HER-2:760-2901, EGFR:790-2988, CK5/6:960-4253, Ki-67:760-2910, p53:760-2912, Ventana Japan, Yokohama, Japan, AR:M3562, Dako Japan, Tokyo, Japan). Visualization of the bound antibodies was performed using a DAKO EnvisionTM + System (Dako Japan Inc., Tokyo, Japan) according to the manufacturer's instructions. Positive

cell rates (%) of ER and PgR were determined as a ratio of positive cells to total cancer cells and a value of 10% or higher were rated as positive [18, 19]. HER-2 expression was defined as (0) to (3+) based on positive cell rates and the intensity of IHC staining. Tumors showing weak overexpression (2+) of HER-2 were also tested by the fluorescence in situ hybridization (FISH) method to clarify the gene amplification of the *HER-2* gene. The *HER-2* gene is visualized as green fluorescent grains and a control of centromere 17 is visualized as orange fluorescent grains (Path Vysion, Abbott, IL, USA). Thus, HER-2 positives were either strong positives (3+) from IHC or positive for gene amplification from FISH analysis.

TNBCs are negative for ER, PgR and HER-2 as described earlier. Among TNBCs with 1–9% of ER and/or PgR expression were defined as hormone receptor (HR) weak and analyzed separately. TNBCs with HER-2 (2+) and that were FISH negative were also analyzed separately.

Proliferative activity was determined by IHC for the Ki-67 antibody. Ki-67 values were expressed as the percentage of positive cell counts among at least 100 tumor cells in each case. Patients with positive staining of Ki-67 at 50% or more were defined as high Ki-67 patients. AR and p53 were defined as positive if tumor cells showed positive staining regardless of rate. Basal-like subtype was defined as CK5/6 positive and/or EGFR positive in 5% or more cells.

Surgical treatment

All patients underwent surgical treatment after NAC. Breast conservative therapy or a mastectomy with or without axillary dissection was performed according to the decision of the surgeons' conference. Surgical specimens were histologically analyzed again, and the pathological response for NAC was evaluated. When no residual invasive tumor cells were found, tumors were identified as pathological complete response (pCR). Surgical specimens from non-pCR patients were analyzed for expressions of Ki-67, p53 and AR as described earlier.

Statistics

A univariate analysis of the pCR rate was carried out by the χ^2 test, and a multivariate analysis was done by multiple logistic regression analysis. The patients' survival was calculated from the first date of treatment until the date of death or the end of follow-up. A univariate analysis of disease-free survival (DFS) was done using the Kaplan–Meier method with a log-rank test, and a multivariate disease survival analysis was carried out under the Cox proportional hazards model. All data were analyzed with JMP for Windows (SAS Institute, Tokyo, Japan).

Results

Relationship between pCR and clinicopathological factors

Thirty-three patients were identified as TNBCs, and the patients' data are shown in Table 1. The age of the patients ranged from 30 to 68 years old (median 50.0) and 21 patients had clinically positive nodes. Clinical response after NAC was rated as clinical complete response for 14 patients (42%), a clinical partial response for 14 patients (42%), a clinical stable disease for 3 patients (9%), and as a clinical progress disease for 2 patients (6%). Also pCR was achieved in only 12 patients (36%).

The correlations between clinicopathological factors such as tumor size, lymph nodal metastasis, age, histological grade, and pCR rate were analyzed (Table 2). However,

Table 1 Patients' characteristics

Variables	No (%)
Total	33
Age: years-old	30–68 (50 ± 11.1)
Histology	
Papillo-tubular	4 (12)
Solid tubular	14 (42)
Schirrous	11 (33)
Special type	4 (13)
T	
1	1 (3)
2	24 (72)
3	6 (18)
4	2 (6)
N	
0	12 (36)
1	17 (52)
2	4 (12)
Histological grade	
1	1 (3)
2	4 (12)
3	27 (81)
Unknown	1 (3)
HER-2	
0	18 (55)
1+	11 (33)
2+	4 (12)
HR (hormone receptor)	
Negative	26 (79)
Weak	7 (21)

T and *N* were defined by the criteria of UICC-breast

HR weak is a tumor with low levels of ER and/or PgR determined by IHC (1–9% weakly positive cells)

Table 2 pCR ratio based on clinicopathologic and immunohistochemistrical factors

Variables	Number (%)	pCR (%)	*P* volume	Odd
Age (years old)				
<50	18 (55)	6 (33)	0.69	
50≤	15 (45)	6 (40)		
Size (cm)				
<5	25 (76)	11 (44)	0.09	5.5
5≤	8 (24)	1 (13)		
N				
Positive	21 (64)	8 (38)	0.78	
Negative	12 (36)	4 (33)		
Histological grade				
1–2	5 (15)	3 (60)	0.26	
3	27 (84)	9 (33)		
HR				
Negative	26 (79)	10 (38)	0.95	
Weak	7 (21)	2 (28)		
HER-2				
0, 1+	29 (88)	9 (31)	0.08	6.67
2+	4 (12)	3 (75)		
p53				
Positive	21 (64)	8 (38)	0.78	
Negative	12 (36)	4 (33)		
Ki-67				
50≤ (high)	20 (61)	10 (50)	*0.04	5.5
<50 (low)	13 (39)	2 (15)		
AR				
Positive	6 (18)	3 (50)	0.45	
Negative	27 (82)	9 (33)		
Basal-like[#]				
Positive	22 (67)	5 (23)	*0.02	5.9
Negative	11 (33)	7 (64)		
CK5/6				
Positive	14 (42)	2 (14)	*0.02	
Negative	19 (58)	10 (53)		
EGFR				
Positive	18 (55)	4 (22)	0.06	
Negative	15 (45)	8 (53)		

* Statistically significant

[#] Basal-like subtype is defined as CK5/4 positive and/or EGFR positive. Thus, CK5/6 was not used for multivariate analysis

these clinicopathological factors did not show any correlation with the pCR rate.

Relationship between pCR, and molecular markers

Next, the correlation between molecular markers and the pCR rate was also analyzed. HER-2 (2+) tended to show a higher pCR rate than HER-2 negative (0 or 1+; 75 and 31%, respectively). In this study, basal markers of CK5/6 and EGFR were evaluated with 22 of 33 patients (67%) diagnosed with basal-like phenotype, and eleven patients (33%) diagnosed with the non-basal-like phenotype. The pCR rate for the basal-like phenotype was significantly lower than in the non-basal-like phenotype (23 and 64%, respectively: $P = 0.02$; Table 2). Ki-67 was also considered as a predictive factor for NAC response, because the pCR rate reaches 50% among high Ki-67 (≥50%) patients, while it was 15% in low Ki-67 patients ($P = 0.04$). The expressions of HR, p53 and AR were not correlated with pCR in this study. Multivariate analysis showed that only high Ki-67 was a significant factor for the prediction of pCR (Table 3). The classification of basal-like or non-basal-like phenotypes was negative for multivariate analysis, probably because high Ki-67 and non-basal-like were strongly correlated with each other; high Ki-67 accounted for 33% in the basal-like and 75% in the non-basal-like phenotype.

Relationship between pCR and disease-free survival

All patients underwent surgical resection after NAC and non-pCR patients were histologically evaluated. The average observation period after surgery was 2 years and eight patients (24%) showed distant metastasis during the observation period. Seven out of 8 patients had been defined as non-pCR and only one patient obtained pCR after NAC. Non-pCR patients showed a worse DFS compared with pCR patients, but it was not statistically significant (Fig. 1a). Basal-like phenotype and other clinicopathological factors such as age, tumor size and lymph nodal involvement failed to show a correlation with DFS (Table 4). Ki-67 before NAC showed a significant correlation with DFS and high Ki-67 patients showed a poor prognosis (Fig. 1b).

Disease-free survival among non-pCR patients

Among non-pCR patients, only 7 patients (29%) showed a recurrence. We analyzed clinicopathological and IHC factors for better prognosis among non-pCR patients. The immunohistological changes of tumors after NAC were

Table 3 Multivariate analysis of pCR and immunopathological factors

Variables	Odds	*P* value
Non-basal-like	3.9	0.13
HER2 (2+)	10.2	0.12
High Ki-67	8.4	0.03*

* Statistically significant

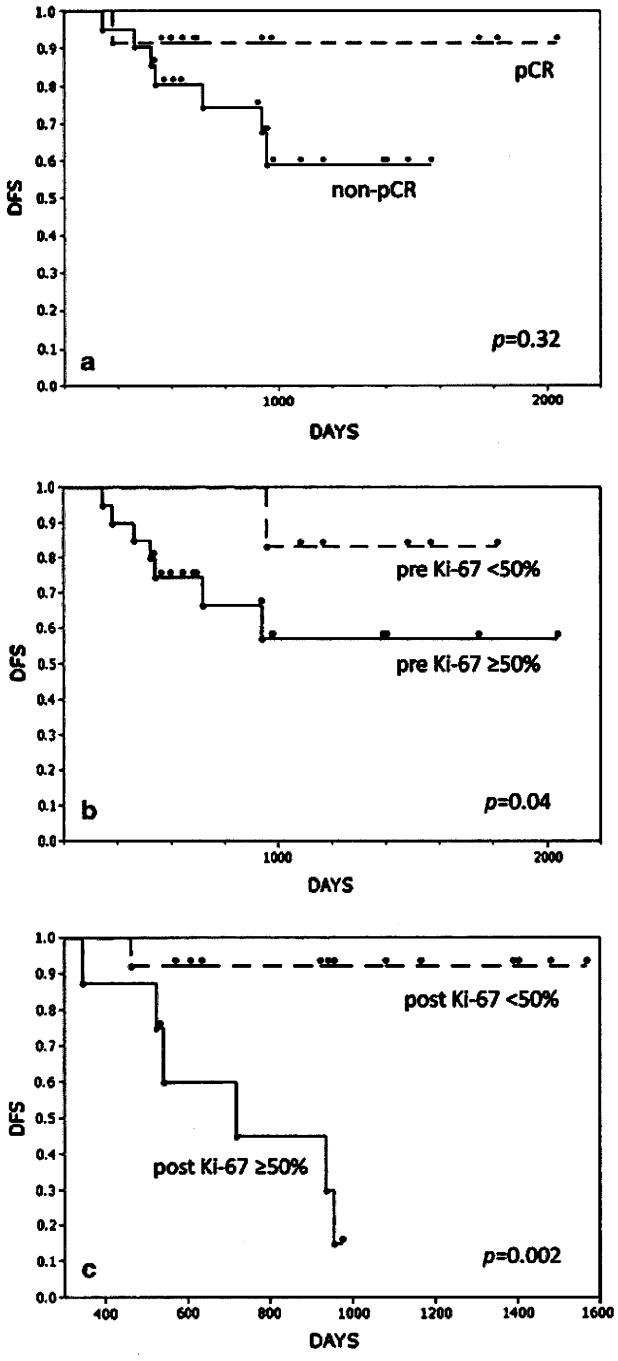

Fig. 1 Disease-free survival (DFS). **a** DFS of pCR and non-pCR patients after NAC. Non-pCR patients showed worse disease-free survival compared with pCR patients, but it was not statistically significant ($P = 0.32$). **b** DFS based on Ki-67 expression of pre-chemotherapy. High Ki-67 (≥50%) patients showed significantly worse disease-free survival than low Ki-67 (<50%) patients ($P = 0.04$). **c** DFS based on Ki-67 expression of post-NAC among non-pCR patients. Non-pCR patients who had high Ki-67 expression after NAC showed a poor prognosis ($P = 0.002$)

evaluated. Among non-pCR patients, 10 patients showed high Ki-67 before chemotherapy and 7 patients still showed high Ki-67 after NAC (Table 5). Among these patients, 6 showed a recurrence and Ki-67 values after NAC were significantly correlated with DFS (Fig. 1c). The expressions of p53 and AR after NAC were not correlated with DFS (data not shown).

Table 4 Multivariate analysis of disease-free survival and patients' characteristics

Variables	Hazard ratio	*P* value
≥50 years-old	0.39	0.2
≥5 cm	2.2	0.3
N positive	4.2	0.11
HR positive	3.2	0.1
HER-2 (2+)	3.2	0.56
Non-basal	1.4	0.6
High Ki-67	5.95	0.04*
p53 positive	0.48	0.3
AR positive	0.000	0.054
Non-pCR	3.7	0.16
High Ki-67 post-NAC#	13.2	0.0029*

Data among non-pCR patients

* Statistically significant

Table 5 The correlation between Ki-67 expression, pCR and the change of Ki-67 expression among non-pCR patients

TNBC ($n = 33$)	Non-pCR		pCR
	Post-NAC Ki-67		
	High	Low	
Pre-NAC Ki-67			
High	7	3	10
Low	1	10	2

Discussion

TNBC is defined by the lack of ER, PgR and HER-2 expression. Because targeted therapies are not useful, chemotherapy is the only systemic treatment option for TNBC [1–5]. Thus, a comprehensive examination of the clinical phenotypes of TNBCs which respond to chemotherapy is important. TNBCs are a heterogeneous group and generally divided into two subtypes; basal-like phenotype and non-basal-like phenotype [6]. The basal-like phenotype is characterized as having a high expression of keratins, laminin, and EGFR.

Many data indicated that the pCR rate is higher in TNBC compared with other phenotypes [10]. A pathological evaluation after NAC is very important because pCR after NAC indicates better survival [8, 9]. Our data showed the pCR rate in TNBCs was 36%, which is consistent with previous

reports which stated 22–45% [10, 20]. This study hypothesized that non-basal-like phenotype, HER-2 (2+), and high Ki-67 could be predictive factors for pCR achievement, but multivariate analysis revealed that only Ki-67 was a significant factor for the prediction of pCR. This is probably because the non-basal-like phenotype showed a significantly higher Ki-67 expression compared with the basal-like phenotype. This study is consistent with previous studies which showed that Ki-67 indicates proliferation and high level of proliferation activity are associated with chemosensitivity [14]. Additionally, there are many reports that showed that the basal-like phenotype has a positive correlation with pCR [20]. Rouzier et al. reported that basal-like subtypes were more sensitive to NAC than luminal and normal-like cancers, but normal-like subtypes classified based on gene expression profiles are quite different from non-basal-like phenotypes based on IHC, because normal-like subtypes involved 60% of ER positive samples. Because classification based on gene expression is difficult for clinical use, our data based on IHC classification are quite useful. There are some reports that non-basal-like tumors showed better prognosis than basal-like phenotypes [6, 7]. Though the pCR rate was significantly higher in non-basal-like tumors, there was no difference in DFS between the two groups in this study.

Our study failed to show the significant benefit of pCR on DFS. That is probably because of the small number of the patients included or the short duration after surgical treatment in this study. Most cases which showed a recurrence in such a short period were non-pCR patients, and the only recurrent case in the pCR group was a patient with an intraductal residual after NAC and who showed brain metastasis within a year. In this study, Ki-67 was the only significant factor which was proved to affect DFS. Pre-NAC high Ki-67 was a poor prognostic factor in spite of the positive correlation with pCR. The post-NAC status of Ki-67 was also correlated with recurrence. High Ki-67 expression post-NAC showed a very poor prognosis and low Ki-67 post-NAC showed better survival even in the non-pCR group. The contradiction of high Ki-67 tumors, which showed a high chemosensitivity and high pCR rate but poor prognosis, may indicate the diversity of these tumors. As shown in Table 4, most high Ki-67 patients who could not achieve pCR kept a high expression of Ki-67 after NAC. Tumors which maintained high Ki-67 expression may indicate that the cellular activity is not suppressed by NAC. All of these facts showed that high Ki-67 tumors should be divided into two groups: tumors which show a high sensitivity to current chemo-drugs and a good prognosis and the tumors which continue to have high cellular activity after NAC and show a poor prognosis. Further study is needed to find other treatments for the latter.

Though many reports defined 20–30% of Ki-67 labeling index as a threshold [21], 50% was used for categorization in this study because most TNBCs are positive for Ki-67 and a 50% threshold at 50% was shown to be useful to predict both chemosensitivity and prognosis in TNBC patients.

The prognosis of HER-2 positive breast cancer has been proved by the usage of trastuzumab. The criteria of HER-2 positive are defined as a strong positive IHC or gene amplification in FISH [22]. HER-2 (2+) breast cancers without gene amplification are generally included in TNBC but HER-2 (2+) breast cancers showed higher chemosensitivity in this study and HER-2 (3+) breast cancers have been reported to be chemosensitive. The criteria of HER-2 positivity might be a moot point if TNBCs with HER-2 (2+) show a different cancer biology from TNBCs with negative HER-2.

Less than 10% of hormone receptor positivity had been considered as uncertain endocrine responsiveness or potential resistance [18, 19]. Though tumors with less than 10% hormone receptor positivity were included in TNBCs, we classified those with 0% staining both ER and PgR as HR negative and those with 1–9% as HR weak in this study. But the expressions of HR were not correlated with pCR. Moreover, tumors with any ER positive staining of at least 1% are recommended to be treated with endocrine therapy in latest reports [21, 23]. The categories of highly endocrine responsive and incompletely endocrine responsive are not relevant to the decision for endocrine therapy, but those categories are still important for the decision of chemotherapy.

In this study, we found that the pCR rate for the non-basal-like phenotype was significantly higher than that in the basal-like phenotype, though that difference was negative for multivariate analysis. This is because the positivity of Ki-67 was higher in the non-basal-like phenotype tumors. These data based on classification by IHC are very interesting and informative in a clinical setting because there are some discrepancy between criteria by gene expression profiling and those by IHC. Some previous papers were confused about classification by gene expression and by IHC. Non-basal-like subtype is a term correlated with IHC classification and difficult to adapt to criteria of gene expression. There are few reports focused on the non-basal-like phenotype. Our data may insinuate that non-basal-like subtypes are well adaptive to current chemotherapy and basal-like subtypes need another therapeutic agent. Because our data was based on a small number of patients, further examinations based on IHC classification are needed.

Our study indicated that TNBCs which were found to be non-pCR with high Ki-67 expression after NAC had a poor prognosis. How to treat these TNBCs will be a most important subject for future study. Only chemotherapy is a

proven treatment for TNBCs, but chemotherapy based on anthracyclins and taxanes has not been shown to be enough. There are several studies which showed the efficacy of new chemotherapeutic agents such as carboplatin, bavasituzumab and poly (ADP-ribose) polymerase-1 (PARP-1) inhibitor in TNBCs [24–26]. Studies of NAC with these agents are expected to improve the treatment of TNBCs.

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Review Article

Epithelial–mesenchymal transition in cancer development and its clinical significance

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The epithelial–mesenchymal transition (EMT) plays a critical role in embryonic development. EMT is also involved in cancer progression and metastasis and it is probable that a common molecular mechanism is shared by these processes. Cancer cells undergoing EMT can acquire invasive properties and enter the surrounding stroma, resulting in the creation of a favorable microenvironment for cancer progression and metastasis. Furthermore, the acquisition of EMT features has been associated with chemoresistance which could give rise to recurrence and metastasis after standard chemotherapeutic treatment. Thus, EMT could be closely involved in carcinogenesis, invasion, metastasis, recurrence, and chemoresistance. Research into EMT and its role in cancer pathogenesis has progressed rapidly and it is now hypothesized that novel concepts such as cancer stem cells and microRNA could be involved in EMT. However, the involvement of EMT varies greatly among cancer types, and much remains to be learned. In this review, we present recent findings regarding the involvement of EMT in cancer progression and metastasis and provide a perspective from clinical and translational viewpoints. (*Cancer Sci* 2010; 101: 293–299)

Development of distant metastases is the final stage of solid cancer progression and is responsible for the majority of cancer-related deaths.[1] Distant metastasis alone or with concurrent locoregional recurrence accounts for nearly 80% of all first relapses in women with breast cancer.[2] While clinically of great importance, the biology of metastasis remains unsolved. The process of tumor metastasis consists of multiple steps, all of which are required to achieve tumor spreading.[3,4] First, cancer cells escape from the primary tumor site. Next, cancer cells invade the tumor stroma and enter the blood circulation directly or the lymphatic system via intravasation. Most circulating cancer cells undergo apoptosis due to anoikis conditions.[5] If cancer cells survive in circulation they may reach more suitable sites by attaching to endothelial cells and extravasating from the circulation into the surrounding tissues. Finally, distal colonization requires that cancer cells invade and grow in the new environment.

Recently, the concept of the epithelial–mesenchymal transition (EMT), as developed in the field of embryology, has been extended to cancer progression and metastasis.[6,7] *In vitro* and experimental animal model data now support the role of EMT in metastasis, concepts supported by analyses of clinical samples. Indeed, the biology of EMT has been clarified in tumor samples through use of EMT-associated markers, such as mesenchymal-specific markers (i.e. vimentin and fibronectin),[8,9] epithelial specific markers (i.e. E-cadherin and cytokeratin),[10,11] and transcription factors (i.e. SNAIL and SLUG).[12]

Most recently, several intriguing studies have described the novel mechanism underlying EMT activation. In the current study, we will discuss the role of small non-coding RNA (microRNA) in regulating EMT-related genes.[13–15] Furthermore, Mani *et al.* disclosed that EMT could generate breast cancer cells with stem cell-like characteristics.[16] Here, we update and discuss recent progress in studies of EMT. These new data improve our understanding of the mechanisms of cancer progression and metastasis as well as therapy resistance. This new information may lead to development of novel clinical targets and improve the clinical management of cancer patients.

Involvement of EMT in Cancer Progression

In the 1980s, Greenburg and Hey first analyzed EMT-associated changes in cell phenotype and mesenchymal states in adult and embryonic epithelia.[17] EMT and the inverse process of mesenchymal–epithelial transition (MET) are major embryological mechanisms for tissue remodeling, as in gastrulation and segment formation.[18] The process of EMT consists of multiple steps.[19,20] First, cell–cell adhesion disintegrates with the loss of epithelial markers such as E-cadherin and the gain of mesenchymal markers such as vimentin. Next, there is a loss of basoapical polarization and the acquisition of front-rear polarization. Then, the cytoskeleton undergoes remodeling, with changes in cortical actin and actin stress fibers. Finally, cell-matrix adhesion is altered, with activation of proteolytic enzymes such as matrix metalloproteases. Note that the process of metastasis in epithelial cancer also consists of multiple steps.[3,4] That is, cells detach from the primary tumor and invade the surrounding tumor stroma. They subsequently enter into the circulation and reach new metastatic sites. Therefore, the process of EMT during cancer progression and metastasis closely resembles that observed in embryologic development. Accordingly, molecular analyses based on EMT in embryology have been applied to cancer progression.

In the 1990s, accumulating evidence indicated that EMT was associated with cancer progression.[7] Indeed, these transformations may be associated with EMT-related signal pathways during development.[7,21] However, Boyer *et al.* stated that EMT during development depends on additional activities of distinct and specific signaling molecules which are highly controlled spatially and temporally, and which do not occur under normal circumstances. On the other hand, EMT in cancer progression could be due to autonomous oncogenic activation of signaling molecules without additional stimulation.[22] Therefore,

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comparisons of EMT signaling pathways in embryological development and cancer progression may make it possible to identify novel pathways specific to cancer progression and to suggest new therapeutic strategies in cancer therapy.[23]

The Molecular Mechanism of EMT in Cancer Progression

Multiple complex signaling systems are required for induction of EMT because epithelial cells undergoing EMT must undergo both functional and morphologic changes. Studies of the crosstalk among the intracellular signal networks could help us to understand the mechanisms regulating EMT. Here, we discuss the regulation of representative molecules, E-cadherin, a major EMT inducer, transforming growth factor-β (TGF-β) signal pathways, and microRNA regulation reported in recent studies (Fig. 1).

E-cadherin regulation. One of the characteristic findings in EMT is the loss of cell–cell adhesion with diminished expression of E-cadherin. E-cadherin, a calcium-dependent transmembrane glycoprotein expressed in most epithelial tissues, constructs a tight junction which connects adjacent cells. The loss of E-cadherin can lead to tumor progression, metastasis, and poorer prognosis in various human carcinomas.[10,11,24,25] Genetic or epigenetic alterations cause a functional loss of E-cadherin. For instance, mutations in E-cadherin are found in diffuse gastric cancer[26] and lobular breast carcinoma.[27] In addition, hypermethylation of the E-cadherin promoter region is found in various human carcinomas, resulting in frequent loss of E-cadherin expression.[28,29] Interestingly, Graff *et al.* proposed that the degree of methylation of the E-cadherin promoter region during metastatic progression is unstable and heterogeneous.[28] This finding suggests that the loss of E-cadherin by methylation in a primary lesion may drive metastatic progression, indicating that EMT is involved in cancer metastasis. Besides genetic or epigenetic control, E-cadherin is regulated by various signal networks, such as TGF-β signaling and transcription factors as discussed in more detail below.

TGF-β signaling. Miettinen *et al.* first revealed that TGF-β induced EMT in normal mammary epithelial cells.[30] In fact, TGF-β is an important inducer of EMT in cancer progression. However, TGF-β is well known to induce multiple responses in cancer progression.[31] For example, loss of the TGF-β signaling pathway results in the progression of cancer because TGF-β is a strong growth inhibitor.[32] Indeed, Hahn *et al.* reported that mutations in TGF-β and Smad4 give rise to pancreatic cancer[33] and colorectal cancer.[34] On the other hand, TGF-β can protect against apoptosis, and promote angiogenesis and immune suppression.[35] TGF-β induces EMT through multiple signal pathways, including direct phosphorylation of Smad 2 and Smad 3. As shown in Figure 1, TGF-β also activates other EMT-related signal pathways, including integrin, Notch, and Wnt signal pathways, all of which trigger EMT programs.

Transcription factors. Transcriptional repressors of E-cadherin such as zinc finger proteins (ZEB1, ZEB2), bHLH protein (Twist), and the snail family of zinc finger proteins (Snail, Slug) are associated with EMT.[36–40] As shown in Figure 1, various signal pathways such as TGF-β,[20] the Wnt cascade, and PI3K/AKT (phosphatidylinositol 3′ kinase–serine/threonine kinase) axis are connected with these transcriptional repressors of E-cadherin.[41] Recent studies have demonstrated that transcriptional repressors of E-cadherin are regulated by microRNAs as described below. Several transcriptional factors such as Snail, Slug, and Twist are useful markers to predict prognosis in various human carcinomas (Table 1). Peinado *et al.* proposed that E-cadherin repressors might participate in the process of EMT as follows. First, Snail and ZEB2 would initiate down-regulation of E-cadherin. Then, Slug and ZEB1 would maintain repression of E-cadherin.[42] However, the effect of E-cadherin repressors on mesenchymal markers such as vimentin and N-cadherin remains unsolved.

Regulation of EMT by microRNA. Recent studies of small non-coding RNAs are shedding light on the regulation of gene expression and proteins in metastasis. It was shown that miR-10b overexpression is associated with invasiveness and metastatic potential.[43] miR-10b is overexpressed in metastatic breast cancer, and up-regulated by EMT transcription factor Twist. Recent independent studies revealed that the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-205 play critical roles in regulating EMT, targeting the E-cadherin repressors ZEB1 and ZEB2.[13,15] Gibbons *et al.* found that metastasis-prone tumor cells established from

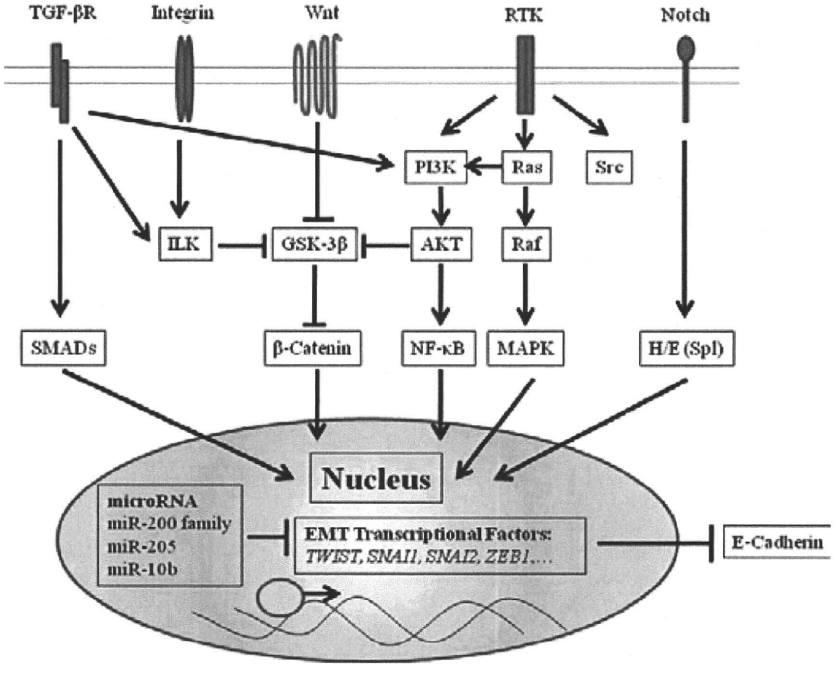

Fig. 1. Depiction of signal pathways regulating the epithelial–mesenchymal transition (EMT). Selected signal pathways regulating E-cadherin are schematized. Transforming growth factor (TGF)-β signals toward the SMAD pathway or the PI3K/AKT axis. Wnt ligands block β-catenin degradation. Excess β-catenin enters the nucleus and upregulates *SLUG* and *SNAIL* transcription. In integrin signaling, overexpression of ILK leads to nuclear translocation of β-catenin. Signals via RTK lead to EMT through the Ras-Raf-MAPK pathway or the PI3K/AKT pathway. AKT, serine/threonine kinase; GSK-3β, glycogen synthase kinase-3β; H/E (Spl), Hairy and enhancer of split; ILK, integrin-linked kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3′ kinase; RTK, receptor tyrosine kinase; TGF-βR, transforming growth factor-β receptor.

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Table 1. Epithelial–mesenchymal transition (EMT)-associated markers in clinical samples predict patient prognosis

EMT-associated gene	Characteristics	Cancer types	Reference (author)
Epithelial marker			
E-cadherin	Type I cell–cell adhesion glycoprotein	Breast cancer	Gould Rothberg and Bracken[25]
		Gastric cancer	Chan *et al.*[24]
		Colorectal cancer	Doridi *et al.*[84]
Claudin-1	Tight junctions restrict lateral diffusion of lipids and membrane proteins	Lung cancer	Chao *et al.*[85]
		Renal cell carcinoma	Fritzsche *et al.*[86]
		Ovarian carcinoma	Kleinberg *et al.*[87]
Mesenchymal marker			
Vimentin	Intermediate filaments represent a third class of cytoskeletal elements	Breast cancer	Thomas *et al.*[88]
		Lung cancer	Al-Saad *et al.*[89]
		Gastric cancer	Utsunomiya *et al.*[90]
N-cadherin	Type I cell–cell adhesion glycoprotein	Esophageal cancer	Yoshinaga *et al.*[91]
		Lung cancer	Nakashima *et al.*[92]
		Urothelial tumor	Lascombe *et al.*[93]
Fibronectin	High-molecular weight extracellular matrix glycoprotein	Bladder tumor	Mutlu *et al.*[94]
		Colorectal cancer	Inufusa *et al.*[95]
		Ovarian carcinoma	Franke *et al.*[96]
Transcription factor			
Snail	Zinc finger transcriptional repressor	Adenocortical carcinoma	Waldmann *et al.*[97]
		Esophageal cancer	Natsugoe *et al.*[98]
		Hepatocellular carcinoma	Miyoshi *et al.*[99]
Slug	Zinc finger transcriptional repressor	Lung cancer	Shih *et al.*[100]
		Colorectal cancer	Shioiri *et al.*[101]
		Esophageal cancer	Uchikado *et al.*[102]
Twist	Basic helix-loop-helix transcription factors	Cervical cancer	Shibata *et al.*[103]
		Ovarian carcinoma	Hosono *et al.*[104]
		Breast cancer	Martin *et al.*[105]

metastatic lung adenocarcinoma (with evidence of mutant K-ras and p53) could transit reversibly between epithelial and mesenchymal states, a property that was regulated by the miR-200 family.[44] Furthermore, two recent independent studies showed that members of the miR-200 family can induce the EMT process and regulate the sensitivity to epidermal growth factor receptor (EGFR) in bladder cancer cells and to gemcitabine in pancreatic cancer cells.[45,46] As for regulating TGF-β, microRNAs related to TGF-β signaling such as miR-155 and miR-29a have been identified in breast cancer tissues.[47,48] It is important to identify microRNAs involved in EMT to elucidate up-stream regulators of various known signal pathways.

Microenvironment and EMT

The tumor microenvironment is composed of the extracellular matrix (ECM), cancer-associated fibroblasts, myofibroblasts, immune cells, and soluble factors required for cancer progression and metastasis. Interaction among cancer cells in the tumor microenvironment can induce EMT by auto- and/or paracrine secretion of mediators such as growth factors, cytokines, and ECM proteins.[21] Media conditioned by cultures of cancer-associated fibroblast induce EMT in breast cancer cells.[49] In a comparison of the central areas of primary colorectal cancer and corresponding metastases, nuclear β-catenin was found in dedifferentiated mesenchyme-like tumor cells at the invasive front and it was localized to the membrane and cytoplasm.[50] This study suggested that the tumor microenvironment may induce or maintain EMT (Fig. 2). For instance, cancer-associated fibroblasts may be supplied from cancer cells undergoing EMT.[51] Similarly, oral squamous cancer cells can directly induce a myofibroblastic phenotype via secretion of TGF-β. TGF-β signaling by stromal myofibroblast can induce secretion of hepatocyte growth factor (HGF) which promotes cancer cell proliferation and invasion.[52]

Drug Resistance and EMT

Cells undergoing EMT become invasive and develop resistance to anticancer agents (Fig. 2). In fact, EMT can be induced by anticancer agents, and stress conditions such as exposure to radiation and hypoxic conditions.[53,54] Up-regulation of *TWIST* was associated with cellular resistance to paclitaxel in human nasopharyngeal, bladder, ovarian, and prostate cancers.[55] In colorectal cancer, stable oxaliplatin-resistant cells established by chronic exposure to oxaliplatin can acquire the ability to migrate and invade with phenotypic changes resembling EMT (spindle-cell shape, loss of polarity, intercellular separation, and pseudopodia formation).[56] In pancreatic and ovarian cancer, stable cell lines resistant to gemcitabine and paclitaxel established by continuous exposure can undergo EMT with increased expression of Snail and Twist, EMT-regulatory transcription factors.[57,58]

Various types of molecularly targeted agents have been developed and used against many carcinomas with or without combination of traditional anticancer agents, leading to improved clinical outcome and survival rate.[59,60] However, EMT reportedly confers resistance to these targeted agents. For example, lung cancer cell lines having undergone EMT, expressing vimentin and/or fibronectin, were insensitive to the growth inhibitory effects of EGFR kinase inhibition (erotinib) *in vitro* and in xenografts[61] as well as other EGFR inhibitors such as gefitinib and cetuximab.[62,63] We have often encountered patients who have suffered relapses after drug treatment, even when the tumors were initially highly sensitive. Thus, EMT can lead to resistance to multiple drugs and permit rapid progression of the tumor. These clinical findings may be attributed to the inherent characteristics of EMT. Clarifying the correlation between EMT and drug resistance may help clinicians select an optimal anticancer drug treatment.

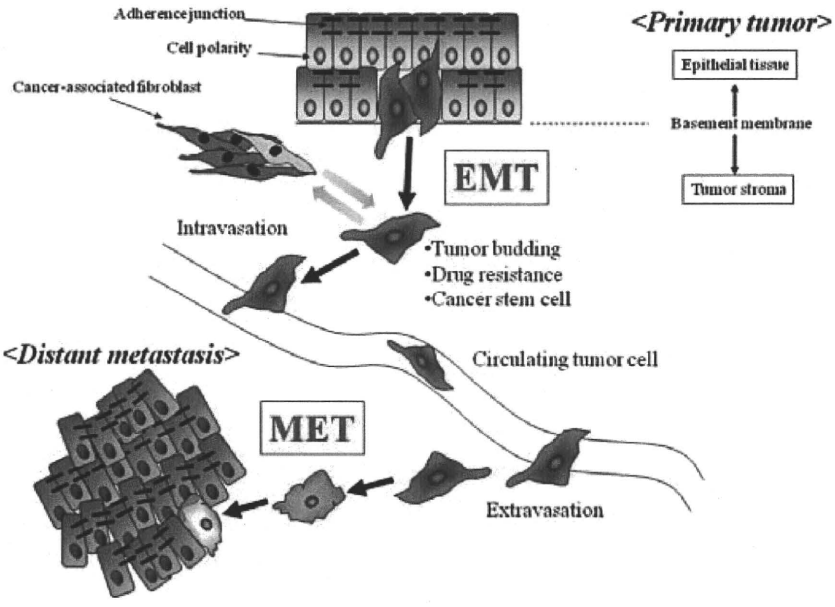

Fig. 2. The epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) are involved in cancer metastasis. Cancer cells undergoing EMT in a primary tumor disseminate through the fragmented basement membrane and acquire the characteristics of drug resistance and cancer stem cells. They can be recognized in tumor buds in histological specimens. EMT cells invade into tumor stroma and enter the circulation, allowing transport to distant organs. At metastatic sites, solitary cancer cells form the new metastatic focus through MET.

Cancer Stem Cells and EMT

Cancer researchers have recently found a minor fraction of cells (cancer stem cells [CSC]) with the ability to self-renew and give rise to differentiated tumor cells. CSC have been identified in breast, colon, and pancreatic cancer.[64–66] CSC as well as cells undergoing EMT are considered to be more resistant to toxic injuries and chemoradiation therapy than differentiated daughter cells.[67,68] Furthermore, cancer cells under hypoxic conditions acquire the properties of CSC.[69,70] Even though evidence indicates a relationship between EMT and cancer cells with the traits of stemness,[71] CSC are rare in whole tumor tissues.[68,72] However, it remains controversial among pathologists whether CSC as well as cells undergoing EMT exist in human cancer tissues.[73] Intriguingly, Mani *et al.* initially disclosed that immortalized human mammary epithelial cells (HMLEs) undergoing EMT are CSC-like as characterized by their $CD44^{high}/CD24^{low}$ phenotype.[16] These investigators induced EMT in HMLEs by ectopic expression of Twist or Snail, known inducers of EMT. The cells undergoing EMT acquired a fibroblastoid mesenchymal appearance. Furthermore, Mani *et al.* observed down-regulation of epithelial markers such as E-cadherin and up-regulation of mesenchymal markers such as N-cadherin, vimentin, and fibronectin. They also noted a $CD44^{high}/CD24^{low}$ expression pattern associated with human breast CSCs. Furthermore, they revealed that the cells undergoing EMT had the properties of CSC, including self-renewal and the capacity to form mammospheres. These findings suggest that EMT may play a role in the development of CSC and properties of invasiveness, metastasis, recurrence, and chemoresistance (Fig. 2).

Clinical Significance of EMT

EMT-associated markers in clinical samples and their effects on prognosis are summarized in Table 1. Most EMT-associated markers have been identified in histological specimens. However, the existence of EMT cells in clinical specimens has been challenged.[74] In response, Voulgari *et al.* suggested that the controversy between experimental and clinical studies is due to the 'spatial' and 'temporal' heterogeneity of EMT (Fig. 3).[19] Cells undergoing EMT may gain metastatic potential but may constitute only a small proportion of the total population of tumor cells. Tumor budding is commonly observed in clinical practice, and it consists of a single cancer cell or small cell cluster at the invasive front of tumor tissues. Indeed, cancer cells in tumor buds have down-regulated E-cadherin[75] and have characteristics of CSC.[76] Therefore, identification of cancer cells undergoing EMT in clinical specimens is difficult for pathologists.

The temporal heterogeneity of EMT (and the reverse, MET) is readily explained. MET is observed *in vitro* following addition of bone morphogenetic protein 7 (BMP7), removal of an EMT-inducer such as TGF-β, and establishment of hypoxic conditions.[54,77] A similar process may occur at metastatic sites which require cancer cells to recover the expression of E-cadherin for cell adhesion. The phenotypes of metastatic specimens are often compared with primary specimens to confirm the diagnosis by hematoxylin–eosin staining. The presence of the same cancer cell characteristics or phenotypes in both primary and metastatic lesions can provide the diagnosis of cancer metastasis. Therefore, the occurrence of MET could make it difficult to prove that EMT, a transient phenomenon that involves only a minority of cells, has occurred in human cancer specimens. However, EMT-associated genes obviously are useful as predictive biomarkers (Table 1). Clinical verification of EMT will require advanced techniques such as *in vivo* imaging.

Treatments Targeting EMT

As shown in Figure 1, EMT-related pathways provide targets for therapy. For instance, inhibition of integrin-linked kinase (ILK) increases the sensitivity of mesenchymal cells to EGFR-target therapy in hepatocellular carcinoma.[63] In *in vitro* studies, Src kinase inhibitors effectively inhibit the growth of cells undergoing EMT.[78] Furthermore, the inhibition of hedgehog signaling can prevent pancreatic cancer cells from acquiring tumor-initiating property and undergoing EMT.[79,80]

RNA interference and microRNA are new technologies in drug development. For instance, silencing of Snail by shRNA induced MET and reduced *in vivo* tumor growth.[81] As for microRNA, Krutzfeldt *et al.* disclosed that specific silencers of endogenous miRNAs, antagomirs, are powerful tools to silence specific miRNAs *in vivo*.[82] Therefore, microRNAs associated with EMT such as the miR-10b and miR-200 family could be exploited as therapeutic strategies in the future.

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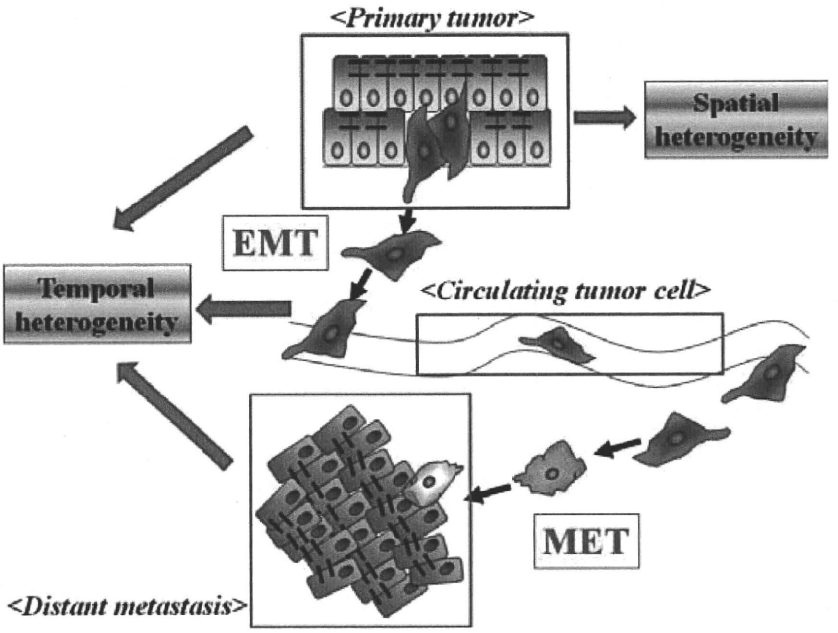

Fig. 3. Spatial and temporal heterogeneity of the epithelial–mesenchymal transition (EMT). Cancer cells undergoing EMT are expected to be only a small proportion of primary tumor tissues. EMT cells transported to metastatic sites are expected to undergo and mesenchymal–epithelial transition (MET). Therefore, the spatial and temporal heterogeneity of EMT/MET severely restricts the ability of pathologists to detect cancer cells undergoing EMT in histological sections.

Furthermore, the tumor microenvironment, which contributes to the maintenance of EMT, could be targeted. A small-interfering RNA targeted at TGF-β reportedly reduces metastasis *in vivo*,[83] and this observation could be applied to TGF-β secreted by tumor stroma. Note that reducing EMT could also lessen the occurrence of anticancer drug resistance and thereby improve the efficacy of conventional therapy. To eradicate cancer cells effectively and cause minimal toxicity to normal cells, further studies are required to define the molecular differences between EMT in embryological development and that in cancer progression.

Perspectives

During the past few decades, an increasing number of studies have shown that EMT is associated with cancer progression, metastasis, and drug resistance. Furthermore, improved understanding of microRNAs and cancer stem cells will clarify the processes underlying EMT. Current understanding of traditional signal pathways coupled with these new concepts could accelerate progress in cancer research. However, the multimodal nature of these complex pathways presents formidable challenges to researchers attempting to inhibit the onset of EMT. Finally, the clinical evidence supporting the role of EMT in cancer progression is still relatively weak. Thus, better methods for EMT detection in patient samples are needed.

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