Table 1 EGFR family tyrosine kinase inhibitors (TKIs) under investigation for the treatment of breast cancer

TKI	Target	Class of action	Phase of study
Gefitinib	EGFR	Reversible TKI	Phase I, II
Erlotinib	EGFR	Reversible TKI	Phase I, II
Aderbasib	EGFR	Reversible TKI	Phase II
AE37	EGFR	Reversible TKI	Phase II
AZD4769	EGFR	TKI	Phase I, solid tumor
Lapuleucel-T	EGFR	Designed to stimulate cellular immune responses against HER2/neu	Phase I
CL-3877785	EGFR	Irreversible TKI	Preclinical
Lapatinib	EGFR, ErbB2	Reversible TKI	In clinical use
BIBW2992	EGFR, ErbB2	Irreversible TKI	Phase II
S222611	EGFR, ErbB2	Reversible TKI	Phase I, solid tumor
TAK285	EGFR, ErbB2	TKI	Phase I, solid tumor
AV412	EGFR, ErbB2	Irreversible TKI	Phase I
PKI-166	EGFR, ErbB2	TKI	Phase I, solid tumor
Varlitinib (ARRY-334543)	EGFR, ErbB2, ErbB4	Reversible TKI	Phase II
BMS-599626	EGFR, ErbB2, ErbB4	Reversible TKI	Phase I
EKB-569	EGFR, ErbB2, ErbB4	Irreversible TKI	Phase I
PF299804	EGFR, ErbB2, ErbB4	Irreversible TKI	Phase I, solid tumor
AZD8931	EGFR, ErbB2, ErbB3	Reversible TKI	Phase I, solid tumor
Vandetanib	EGFR, VEGF, RET	TKI	Phase I, II
CUDC101	EGFR, ErbB2, HDAC	Irreversible TKI	Phase I, solid tumor, phase Ib
Neratinib (HKI-272)	Pan-EGFR	Irreversible TKI	Phase I, II, III
Canertinib (CI-1033)	Pan-EGFR	Irreversible TKI	Phase I, II
BMS690514	Pan-EGFR, VEGFR2	Irreversible TKI	Phase l, solid tumor
XL647	EGFR, ErbB2, EphB4, VEGF	Reversible TKI	Phase I
AEE788	EGFR, ErbB2, VEGFR	Reversible TKI	Phase I
ARRY380	ErbB2, AKT	Reversible TKI	Phase I

EGFR epidermal growth factor receptor, *HDAC* histone deacetylase, *VEGF* vascular endothelial growth factor, *VEGFR2* VEGF receptor 2

Clinical trials of EGFR inhibitors for breast cancer

EGFR inhibitors for treatment of breast cancer have been evaluated in several studies (Tables 3, 4), but results so far have been disappointing.

Gefitinib monotherapy did not significantly improve response rates in most studies [58–60]. In a phase II trial of erlotinib monotherapy in patients with metastatic breast cancer ($n = 69$), no patients had a complete response, and only 2 had a partial response [61]. These rather disappointing results may relate to the patient selection criteria: these trials did not select patients on the basis of EGFR expression; rather, they included unselected breast cancer patients who had often been heavily pretreated. Subgroup analysis of results of previous clinical trials may offer clues to optimal patient selection for EGFR-targeted therapy.

Two phase II clinical trials evaluated the efficacy of cetuximab alone or in combination with platinum-based chemotherapy and found that the addition of cetuximab to carboplatin did not improve outcome [62, 63]. However, in one of these trials [62], cetuximab alone was associated with a 6 % response rate, suggesting that this drug could have some efficacy in selected patients. In that trial, the authors also investigated EGFR pathway activation by gene expression profiling with Agilent DNA microarrays. The findings suggested that cetuximab blocked the expression of the EGFR pathway in only a minority of patients, indicating that most patients had alternate mechanisms of pathway activation [62].

Another phase II trial in patients with advanced breast cancer showed that cetuximab improved the overall response rate only in patients with TNBC; in this subgroup,

Table 2 Monoclonal antibodies (MAbs) against epidermal growth factor receptor under investigation for treatment of breast cancer

MAb	Class of action	Phase of study
Cetuximab	Chimeric MAb	Phase I, II
Panitumumab	Humanized MAb	Phase II
GA 201	MAb	Phase I, solid tumor
Nimotuzumab	Humanized MAb	Phase I
Matuzumab	Humanized MAb	Preclinical

overall response rates were 38 % for patients treated with irinotecan and carboplatin and 49 % for patients treated with irinotecan, carboplatin, and cetuximab [63]. EGFR positivity was not an eligibility criterion for this trial, but retrospective assessment showed that EGFR positivity was significantly associated with the TNBC subtype ($P < 0.0001$).

European researchers were the first to prove that anti-EGFR therapy can provide substantial clinical benefit for patients with TNBC [64]. These researchers conducted a phase II randomized trial of cisplatin versus cisplatin plus cetuximab in 173 heavily pretreated patients with TNBC. The overall response rate was twice as high with the cisplatin-cetuximab combination as it was with cisplatin alone (20 vs. 10.3 %). Also, the median progression-free survival time was more than twice as long with cetuximab as it was with cisplatin alone (3.7 vs. 1.5 months).

Panitumumab, an antibody targeting EGFR, has also been investigated for its efficacy in patients with TNBC. In a phase II trial of panitumumab in combination with 5-fluorouracil, epidoxorubicin, and cyclophosphamide followed by docetaxel for neoadjuvant therapy, the overall response rate was 80 % [65]. A trial of panitumumab in combination with carboplatin for patients with metastatic TNBC is ongoing (NCT00894504).

Taken together, the findings to date on EGFR-targeted agents suggest that further investigation of these agents in patients with TNBC is warranted [66].

Clinical trials of dual EGFR and HER2 inhibitors for breast cancer

In a phase I clinical trial, lapatinib monotherapy showed clinical activity in patients with trastuzumab-refractory breast cancer. Four of the 59 evaluable patients with metastatic breast cancer positive for both EGFR and HER2, including 2 with IBC, had a partial response [67]. In a phase II trial of lapatinib monotherapy for heavily pretreated patients with IBC, the response rate was 50 % among the 30 patients with HER2-positive tumors but only 7 % among the 15 patients with HER2-negative, EGFR-positive tumors [68]. The investigators also evaluated ErbB3 status and found that co-expression of phosphorylated HER2 and phosphorylated ErbB3 was associated with a better response rate. In a phase II trial, BIBW 2992, a novel oral, irreversible EGFR and HER2 inhibitor, had a rate of clinical benefit of 14 % in 21 patients with metastatic TNBC [69]. At present, response to dual EGFR and HER2 inhibitors seems to depend on HER2 expression rather than EGFR expression. Further studies of dual inhibitors are required.

Conclusions

Previous trials showed that many patients with EGFR-expressing tumors did not respond to EGFR-targeted therapy, which suggests that EGFR expression alone does not indicate tumor cell dependence on the EGFR pathway. There is now evidence for significant interactions of EGFR with other receptor tyrosine kinases, such as c-MET and IGF-1R, and it is possible that such alternative signaling pathways are linked to resistance to targeted therapies [39]. Thus, we have to consider combining EGFR-targeted therapy with drugs targeting these alternate signaling pathways to improve efficacy. Further, EGFR activation drives the migration and invasion through EMT and alters chemosensitivity by rewiring the apoptotic signaling network. Therefore, EGFR-targeted therapy may not produce cancer shrinkage due to suppression of cell proliferation; rather, EGFR-targeted therapy may produce a therapeutic effect by inhibiting metastasis or sensitizing cancer cells to the effects of cytotoxic therapy.

Recent studies confirm that EGFR is a potentially important target in breast cancer, especially TNBC, basal-like breast cancer, and IBC. EGFR-targeted therapy has finally shown some promise in terms of improving outcomes in breast cancer patients, but molecular prognostic and predictive factors need to be identified to optimize selection of patients for EGFR-targeted therapies. Mechanistic, hypothesis-oriented clinical trials are needed rather than trials based on the assumption that EGFR-targeted therapy will be effective against EGFR-overexpressing breast cancer. Further, recent data suggest that expecting EGFR-targeted therapy to produce tumor shrinkage may be unrealistic. EGFR-targeted therapy may be most effective as a chemosensitizer or therapy designed to prevent metastases.

Table 3 Clinical trials of EGFR inhibitors as monotherapy

Drug studied	First author, year (Ref)	No. of patients	Type of study	Patient population	Dosage	Response	Patient outcome
Gefitinib	von Minckwitz, 2005 [58]	58	Phase II	Taxane- and anthracycline-pretreated MBC	500 mg/day	PR: 1.7 % (1/58) SD: 0 PD: 89.7 % (52/58) CB: 1.70 % (1/58)	Median time to progression, 61 days (95 % CI: 54–82). EGFR evaluated, no correlation between EGFR expression and response
Gefitinib	Baselga, 2005 [60]	31	Pharmacodynamic, phase II	Advanced breast cancer	500 mg/day	CR: 0 PR: 0 SD: 38.7 % (12/31) PD: 61.3 % (19/31)	Good correlation between degree of inhibition of EGFR in skin and breast tumors. However, lack of EGFR dependence in tested population
Erlotinib	Dickler, 2009 [61]	69	Phase II	Unselected, previously treated MBC.	150 mg/day	PR: 3 % (2/69) SD: 11.6 % (8/69) PD: 79.7 % (55/69)	Erlotinib showed minimal activity
EKB-569	Erlichman, 2006 [70]	Cohort 1: 30 Cohort 2: 29	Phase I	Advanced solid tumors	Escalating doses: 25, 50, 75, 125, 175, and 225 mg. Intermittent dosing or continuous dosing		No major antitumor responses observed. Tolerable toxic effects and long half-life of this irreversible EGFR inhibitor warrant its further evaluation as a single agent and in combination with other drugs
Lapatinib	Burris, 2005 [67]	67 (30 patients with breast cancer)	Phase I	ErbB1- and/or ErbB2-expressing advanced refractory solid tumors	13 pts, 500 mg/day; 15 pts, 650 mg/day; 11 pts, 900 mg/day; 3 pts, 1,000 mg/day; 12 pts, 1,200 mg/day; 13 pts, 1,600 mg/day	PR: 6 % (4/67) (all breast cancer pts) SD: 36 % (24/67) (10 breast cancer pts)	Four patients with trastuzumab-resistant MBC, two of whom were classified as having inflammatory breast cancer, had partial responses
Lapatinib	Johnston, 2008 [68]	45 Cohort A: 30 Cohort B: 15	Phase II	Anthracycline-refractory MIBC. Cohort A: HER2-overexpressing disease Cohort B: HER2-neg, EGFR-pos disease	1,500 mg/day	Cohort A: PR: 23 % (7/30) SD: 23 % (7/30) Cohort B: PR: 7 % (1/15)	Lapatinib well tolerated with clinical activity in heavily pretreated HER2-positive but not EGFR-pos/HER2-neg IBC. Co-expression of pHER2 and pHER3 in tumors seemed to predict for a favorable response

Table 3 continued

Drug studied	First author, year (Ref)	No. of patients	Type of study	Patient population	Dosage	Response	Patient outcome
Lapatinib	Burstein, 2008 [71]	229 Cohort A: 140 Cohort B: 89	Phase II	Previously treated MBC Cohort A: HER2-pos disease Cohort B: HER2-neg disease	1,500 mg/day	Cohort A: CB: 5.7 % (CR, PR, or SD $\geq$ 24 weeks) Cohort B: CB: 0 % Only one patients had SD $\geq$ 16 weeks	Lapatinib had modest clinical activity in HER2-pos MBC that progressed on prior trastuzumab regimens. No apparent clinical activity was observed in chemotherapy-refractory, HER2-neg disease
Canertinib	Calvo, 2004 [72]	24 (1 BC)	Phase I pharmacokinetic	Advanced solid malignancies	Escalating doses		Recommended dose on this schedule is 250 mg/day. No patient had objective evidence of a major response
Canertinib	Nemunaitis, 2005 [73]	32 (3 BC)	Phase I pharmacokinetic	Solid tumors refractory to standard therapy	9 pts: 300 mg/day 3 pts: 350 mg/day 6 pts: 450 mg/day 8 pts: 500 mg/day 6 pts: 560 mg/day	SD: 19 % (6/32)	None of the 3 patients with breast cancer showed a response
BIBW 2992	Yap, 2010 [74]	53 (4 BC)	Phase I	Advanced solid tumors	Escalating doses (10–50 mg/day)	PR: 15 % (5/34) SD: 20 % (7/34)	PR: 4 pts with NSCLC, 1 with esophageal cancer. SD: 1 pt with mesothelioma, 1 with breast cancer, 2 with colorectal cancer, 1 each with cervical cancer, thyroid carcinoma, unknown tumor type
BIBW 2992	Schuler, 2010 [69]	Cohort A: 29 Cohort B: 21	Phase II	Cohort A: HER2-neg, HR-pos MBC Cohort B: TNBC	50 mg/day	Cohort A: PR: 0 % SD: 0 % Cohort B: SD: 14 % (3/21)	Side effects mainly affected the skin and gastrointestinal tract and were manageable. Clinical benefit was achieved in a fraction of pts with TNBC
Cetuximab	Carey, 2008 [62]	102	Phase II	TNBC with MBC, no prior platinum	Cetuximab alone (400 mg/m^2 then 250 mg/m^2 weekly) versus cetuximab + carboplatin	PR: 6 % versus 18 % SD: 4 % versus 9 % CB: 10 % versus 27 % (CB = PR or SD > 6 mo)	Cetuximab alone was well tolerated and produced some responses. This arm was closed for insufficient activity. The cetuximab + carboplatin arm produced higher response and clinical benefit rates

CB clinical benefit, *EGFR* epidermal growth factor receptor, *IBC* inflammatory breast cancer, *MBC* metastatic breast cancer, *neg* negative, *MIBC* metastatic inflammatory breast cancer, *pos* positive, *NSCLC* non-small cell lung cancer, *PD* progressive disease, *PR* partial response, *SD* stable disease, *TNBC* triple-negative breast cancer

Table 4 Clinical trial for EGFR inhibitor: Combination therapy

Drug studied, trial name	First author, year (Ref)	No. of patients	Type of study	Patient population	Combination therapy	Response	Patient outcome
Gefitinib	Polychronis, 2005 [75]	56	Phase II randomized trial	Newly diagnosed postmenopausal, ER-pos, EGFR-pos breast cancer	Arm 1: gefitinib 250 mg/day + anastrozole 1 mg/day Arm 2: gefitinib 250 mg/day + placebo	Arm 1 versus arm 2: CR: 0 versus 0 % PR: 44 % (12/27) versus 48 % (14/29) SD: 37 % (10/27) versus 48 % (14/29) PD: 0 versus 0 %	Tumor size reduction on ultrasonography. Gefitinib either alone or in combination with anastrozole substantially reduced tumor size
Gefitinib	Smith, 2007 [76]	206	Phase II placebo-controlled randomized trial	Newly diagnosed stage I to IIIB HR-pos breast cancer	Arm 1: anastrozole + gefitinib Arm 2: anastrozole to anastrozole + gefitinib Arm 3: anastrozole alone	Anastrozole versus Anastrozole + gefitinib: CR: 4 % versus 7 % PR: 57 % versus 40 % SD: 33 % versus 37 % PD: 4 % versus 5 % CB: 61 % versus 48 %	Study failed
Gefitinib,	Mauriac, 2008 [77]	108	Phase II randomized trial	MBC	Arm 1: anastrozole + gefitinib Arm 2: anastrozole + placebo	PD: 72 % (26/36)	Thirty-six patients completed treatment. The estimated PFS rate at 1 year was 42 %
Gefitinib	Cristofanilli, 2008 [78]	94	Phase II randomized trial	Postmenopausal, newly diagnosed HR-pos MBC	Arm 1: anastrozole + gefitinib Arm 2: anastrozole + placebo	Arm 1 versus arm 2: CR: 2 % (1/43) versus 2 % (1/50) PR: 0 % (0) versus 10 % (10/50) SD: 47 % (20/43) versus 22 % (11/50) CB: 49 % (21/43) versus 34 % (17/50)	Anastrozole + gefitinib was well tolerated and associated with a marked advantage in PFS versus anastrozole + placebo.
Gefitinib, NSABP FB-IR-002	Dennison, 2007 [79]	33	Phase II	MBC	Gefitinib 250 mg/day + docetaxel 75 mg/m^2	CR: 3 % (1/33) PR: 36 % (12/33) SD: 12 % (4/33) PD: 48 % (16/33) CB: 52 % (17/33)	The median duration of CB was 10.9 months (95 % CI, 6.0– 17.6)
Gefitinib, 0024154 ECOG-1100	Arteaga, 2008 [80]	35	Phase II	HER2-pos MBC	Trastuzumab 2 mg/kg/week + gefitinib 250 mg/day or 500 mg/day	CR: 3 % (1/32) PR: 6 % (2/32) SD: 19 % (6/32) PD: 28 % (9/32) CB: 28 % (9/32)	These results do not support the use of this combination in patients with HER2-pos breast cancer

Table 4 continued

Drug studied, trial name	First author, year (Ref)	No. of patients	Type of study	Patient population	Combination therapy	Response	Patient outcome
Erlotinib	Mayer, 2006 [81]	22	Phase II	Postmenopausal HR-sensitive MBC	Letrozole 2.5 mg/day + erlotinib 150 mg/day	CR: 5 % (1/20) PR: 20 % (4/20) SD: 30 % (6/20) PD: 25 % (5/20) CB: 55 % (11/20)	Median time to progression was 13 months. EGFR immunohistochemistry was negative in all cases. Two of 18 patients had HER2-pos disease
Erlotinib	Twelves, 2008 [82]	24	Phase II	MBC	Capecitabine + docetaxel + erlotinib in several dosages.	CR: 8 % (2/24) PR: 50 % (12/24) SD: 21 % (5/24) PD: 8 % (2/24)	Recommended for further study is erlotinib 100 mg/day continuously with capecitabine 825 mg/m^2 bid and docetaxel 75 mg/m^2 intravenously. The exposure to the three drugs is not diminished when they are given in combination
Erlotinib	Venturini, 2004 [83]	9	Phase II dose-finding study	MBC	Erlotinib 50, 100 mg/day, or 150 mg/day sequentially after capecitabine + vinorelbine for 6 cycles or 8 cycles	PR: 50 % (3/6) SD: 33 % (2/6) PD: 17 % (1/6)	Established maximum tolerated dose
Erlotinib	Kaur, 2006 [84]	31	Phase II	MBC	Docetaxel 35 mg/m^2 weekly + erlotinib 150 mg/day	PR: 55 % (11/20) SD: 35 % (7/20) PD: 10 % (2/20)	Promising activity with favorable response compared to other studies
Erlotinib	Dickler, 2008 [85]	38	Phase II	MBC	Erlotinib 150 mg + bevacizumab 15 mg/kg intravenously every 3 weeks	PR: 2.7 % (1/37) SD: 51.3 % (19/37) PD: 51.3 % (19/37)	EGFR expression was not predictive of response to therapy
Erlotinib	Beeram, 2005 [86]	16 (14 patients with breast cancer)	Phase I	Advanced solid tumors	Erlotinib 50, 100 mg/day, or 150 mg/day + trastuzumab 2 mg/kg/week + paclitaxel 80 mg/m^2		1 CR, 2 PRs, 1 minor response observed in patients with breast cancer in whom prior trastuzumab therapy failed
Lapatinib	Johnston, 2009 [87]	1286	Phase III randomized trial	Postmenopausal, HR-pos MBC	Arm A: letrozole 2.5 mg/day + lapatinib 1500 mg/day Arm B: letrozole 2.5 mg/day + placebo	HER2-pos patients, arm A versus arm B: CR: 5 versus 4 % PR: 23 versus 11 % SD: 20 versus 14 % CB: 48 versus 29 % HER2-neg patients, arm A versus arm B: CR: 5 versus 4 % PR: 28 versus 27 % SD: 26 versus 25 % CB: 58 versus 56 %	In HER2-pos patients ($n = 219$), addition of lapatinib to letrozole significantly reduced the risk of disease progression (hazard ratio = 0.71; 95 % CI, 0.53–0.96; $P = 0.019$). In HER2-neg patients ($n = 952$), no improvement in PFS was observed with addition of lapatinib

Table 4 continued

Drug studied, trial name	First author, year (Ref)	No. of patients	Type of study	Patient population	Combination therapy	Response	Patient outcome
Lapatinib	Di Leo, 2008 [88]	579	Phase III randomized trial	HER2-neg or HER2-untested MBC	Arm A: paclitaxel 175 mg/m^2 every 3 weeks + lapatinib 1500 mg/day Arm B: paclitaxel 175 mg/m^2 every 3 weeks + placebo	HER2-pos patients, arm A versus arm B: CR: 10 versus 3 % PR: 53 versus 35 % SD: 18 versus 30 % PD: 14 versus 22 % CB: 69.4 versus 40.5 % $P = 0.011$ HER2-neg patients, arm A versus arm B: CR: 3 versus 2 % PR: 27 versus 21 % SD: 34 versus 46 % PD: 25 versus 25 % CB: 4.7 versus 31.9 % $P = 0.806$	Neither patients with HER2-neg disease nor those with HER2-untested disease benefited from the addition of lapatinib to paclitaxel
Canertinib	Garland, 2006 [89]	26	Phase I	Advanced solid tumors	Canertinib 50–75 mg/day + docetaxel 75 mg/m^2	CR: 4.7 % (1/21) PR: 4.7 % (1/21) SD: 4.7 % (1/21) PD: 85.7 % (18/21)	Resulted in a recommended phase II dose of canertinib 50 mg/day + docetaxel 75 mg/m^2
Cetuximab	Modi, 2006 [90]	12	Phase I dose-escalation study	EGFR-pos MBC	Cetuximab + paclitaxel	SD: 17 % (2/12) PD: 67 % (8/12)	Because of prohibitive dermatologic toxic effects and disappointing preliminary efficacy, the combination was not considered promising in this population
Cetuximab	Baselga, 2010 [64]	173	Phase II	TNBC or MBC	Arm 1: cetuximab 400 mg/m^2 initial dose then 250 mg/m^2 weekly + cisplatin 75 mg/m^2 Arm 2: cisplatin alone (after 6 cycles could switch to cetuximab + cisplatin)	Overall response rate, arm 1 versus arm 2: 20.0 versus 10.3 %	Adding cetuximab to cisplatin nearly doubled the overall response rate. Cetuximab + cisplatin was associated with a significant 32.5 % reduction in the risk of disease progression compared with cisplatin alone (hazard ratio = 0.675; $P = 0.032$)
Cetuximab, USOR04-070	O'Shaughnessy, 2010 [63]	154	Phase II randomized trial	MBC	Arm 1: irinotecan + carboplatin Arm 2: irinotecan + carboplatin + cetuximab		Cetuximab did not improve antitumor activity, but subset analysis showed that the addition of cetuximab increased the overall response rate associated with irinotecan + cisplatin in TNBC (49 vs 38 %)

Table 4 continued

Drug studied, trial name	First author, year (Ref)	No. of patients	Type of study	Patient population	Combination therapy	Response	Patient outcome
Cetuximab	Rivera, 2011 [91]	43	Retrospective	Advanced TNBC	Cisplatin 50 mg/m^2 or carboplatin + cetuximab 400 mg/m^2 initial dose then 250 mg/m^2 weekly	Objective response rate: 45.9 %	Median treatment duration was 2.3 months. The objective response rate was 45.9 % among 37 evaluable patients. Median time to progression was 2.3 months (95 % CI = 1.9–4.0)
Panitumumab	Nabholtz, 2011 [65]	58	Pilot phase II	Newly diagnosed TNBC	FEC100 (fluorouracil 500 mg/m^2 + epirubicin 100 mg/m^2 + cyclophosphamide 500 mg/m^2) every 3 weeks for 4 cycles followed by docetaxel 100 mg/m^2 every 3 weeks for 4 cycles + panitumumab 9 mg/kg every 3 weeks for 8 cycles	Pathologic CR: 17 patients Overall clinical response rate: 80 %	Preliminary results suggest that panitumumab in combination with FEC100 followed by docetaxel appears efficacious with acceptable toxicity for neoadjuvant therapy for operable TNBC

bid twice a day, *CB* clinical benefit, *CR* complete response, *ECOG* Eastern Cooperative Oncology Group, *EGFR* epidermal growth factor receptor, *ESMO* European Society for Medical Oncology, *HR* hormone receptor, *neg* negative, *NSABP* National Surgical Adjuvant Breast and Bowel Project, *PD* progressive disease, *PF*, progression-free survival, *pos* positive, *PR* partial response, *SD* stable disease, *TNBC* triple-negative breast cancer, *USOR* US Oncology Research

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A Radial Sclerosing Lesion Mimicking Breast Cancer on Mammography in a Young Woman

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Key Words
Breast cancer · Radial sclerosing lesion · Mammography · Young women

Abstract
A spiculated mass on a mammogram is highly suggestive of malignancy. We report the case of a 32-year-old woman with a radial sclerosing lesion that mimicked breast cancer on mammography. She visited her physician after palpating a lump in her left breast. Mammography showed architectural distortion in the upper inner quadrant of the left breast. Ultrasonography showed a low echoic area with an ambiguous boundary. Core needle biopsy was performed because of the suspicion of malignancy. Histological examination did not reveal any malignant cells. After 6 months, the breast lump became larger and the patient was referred to our hospital. Mammography performed in our hospital showed a spiculated mass, and therefore mammotome biopsy was performed. Histological examination revealed dense fibroelastic stroma with a wide variety of mastopathic changes, leading to a diagnosis of a radial sclerosing lesion. One year after the biopsy, the lump on her left breast had disappeared and mammography showed no spiculated mass.

Introduction

Mammography is the most sensitive method for detecting breast lesions, but it lacks specificity. Benign and malignant breast lesions often present with overlapping mammographic findings, and thus a definite radiologic diagnosis may be difficult to

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establish [1]. Spiculated breast lesions, which reflect a wide variety of pathologic entities, are detected relatively frequently on routine mammograms. Here, we report the case of a young woman in whom a radial sclerosing lesion mimicking breast cancer was detected on mammography.

Case Presentation

A 32-year-old woman visited her physician after she had palpated a lump in her left breast. Mammography showed architectural distortion in the upper inner quadrant of the left breast. Ultrasonography showed a low echoic area with an ambiguous boundary (fig. 1). A core needle biopsy was performed because of suspicion of malignancy. Histological examination did not reveal any malignant cells. Six months after the core needle biopsy, the breast lump became larger and the patient was referred to our hospital. Mammography performed in our hospital showed architectural distortion in the upper inner quadrant of the left breast in a medial lateral oblique view (fig. 2a) and a spiculated mass on the craniocaudal spot in a compression magnification view (fig. 2b). Mammotome biopsy was performed because of a strong suspicion of malignancy. Histological examination of the mammotome biopsy revealed a sclerotic area consisting of dense collagenous tissue and entrapped benign ducts. A wide variety of mastopathic changes such as duct hyperplasia, apocrine cysts, sclerosing adenosis, and microcystic deletion of glands were also seen. These findings led to diagnosis of a radial sclerosing lesion (fig. 3). One year after the mammotome biopsy, the lump on her left breast had disappeared and mammography showed no spiculated mass (fig. 2c).

Discussion

The Breast Imaging Reporting and Data System (BI-RADS) developed by the American College of Radiology provides a standardized classification for mammographic studies. Mammograms in BI-RADS category 5 indicate a strong suspicion of malignancy. The specific mammographic features with the highest positive predictive value for malignancy include masses with spiculated margins and/or irregular shapes, as well as calcifications with a linear morphology and/or segmental distribution [2]. The positive predictive value of a biopsy positive for malignancy is 95–97% for category 5 mammograms [2, 3].

Many benign breast lesions, such as abscess, hematoma, radial sclerosing lesion, postsurgical scar, diabetic mastopathy, focal fibrosis, sclerosing adenosis, granular cell tumor, extra-abdominal desmoid tumor, and medial insertion of pectoralis and sternalis muscles, pose diagnostic challenges [1, 4]. Radial sclerosing lesions are benign pseudo-infiltrative lesions characterized by a central zone of fibroelastosis from which epithelial structures radiate out in a stellate formation [5, 6]. The investigation and treatment of these lesions can be difficult because they resemble infiltrating carcinomas radiologically, as well as histologically, due to their similarity to well-differentiated carcinomas [5–7]. Radial sclerosing lesions show the following characteristics on mammograms: (a) varying appearance in different projections, with no dense solid center, rather than a translucent center; (b) longer and thinner radiating spicules; (c) radiolucent linear structures parallel to spicules, and (d) absence of a palpable lesion or skin changes [6]. The association of these lesions with significant proliferative changes and, in particular, with preneoplastic conditions is becoming increasingly apparent [5, 8, 9].

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Mammotome biopsy is the method of choice for evaluation of suspicious lesions, since it has been proven to be safe without significant complications, to be easy to perform with high adaptability, and to have excellent patient acceptance. With the advent of the mammotome method many breast lesions can be removed in a breast radiology department, and further excision can be performed if histology shows associated ductal carcinoma in situ or invasive cancer [10]. In our case, the tumor shrank and disappeared on a mammogram 1 year after mammotome biopsy; however, the patient should still be followed up carefully in this type of case. Although a spiculated mass on a mammogram is highly suggestive of breast cancer, our case indicates that a radial sclerosing lesion should be considered as a differential diagnosis.

Disclosure Statement

The authors have no conflicts of interest to disclose.

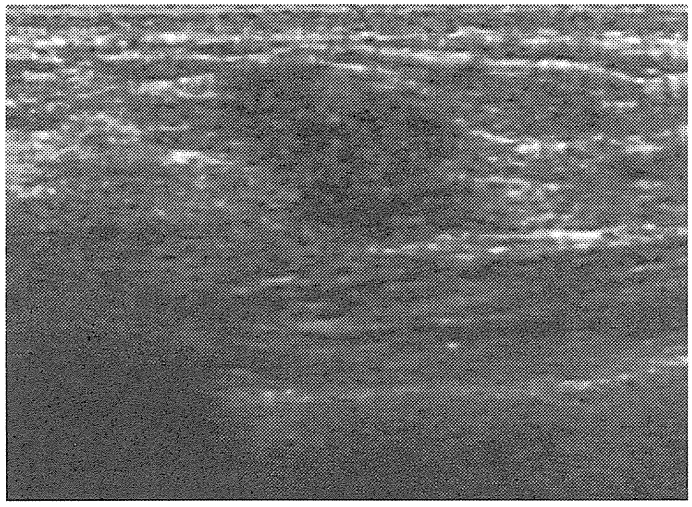

Fig. 1. Ultrasonogram showing a low echoic area with an ambiguous boundary.

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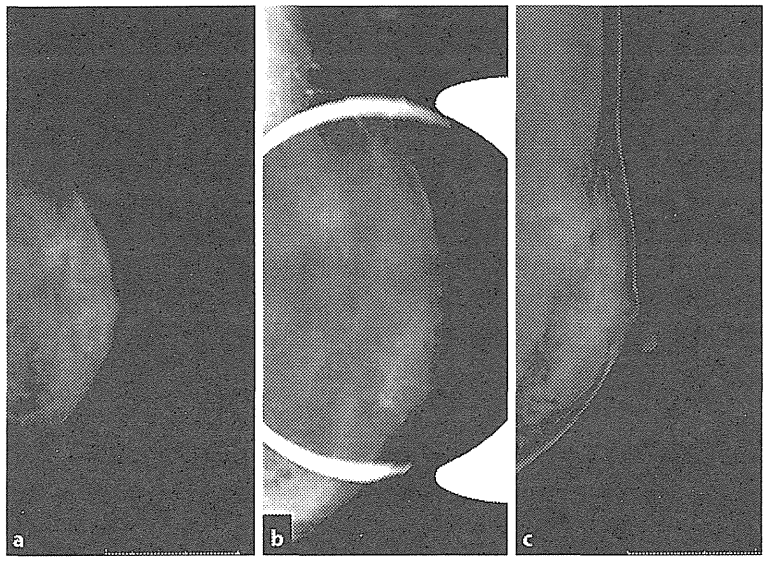

Fig. 2. Mammographic findings. Before mammotome biopsy, architectural distortion in the upper inner quadrant of the left breast was evident in a medial lateral oblique view (**a**) and a spiculated mass was seen on the craniocaudal spot in a compression magnification view (**b**). One year after mammotome biopsy, the spiculated mass had disappeared (**c**).

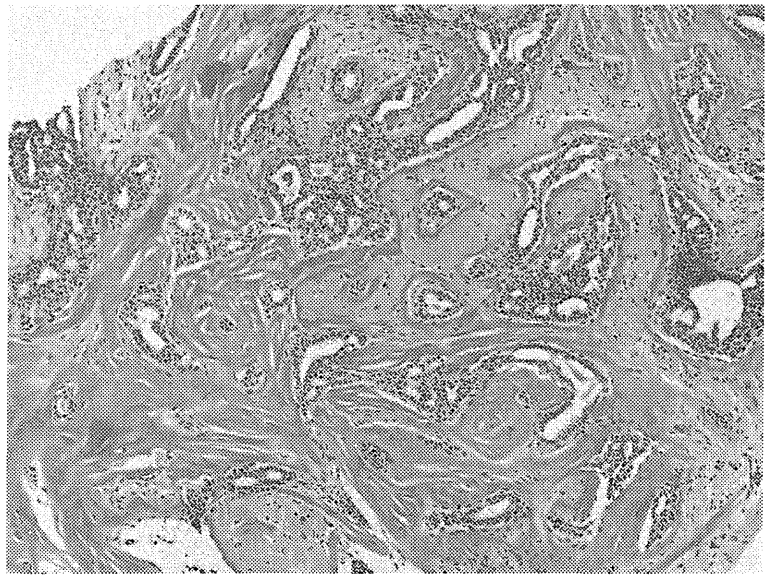

Fig. 3. Mammotome biopsy showing fibroelastic stroma with entrapped small ducts and duct hyperplasia.

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ORIGINAL PAPER

DNA methylation status of *REIC/Dkk-3* gene in human malignancies

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Abstract
Purpose The *REIC (reduced expression in immortalized cells)/Dkk-3* is down-regulated in various cancers and considered to be a tumor suppressor gene. *REIC/Dkk-3* mRNA has two isoforms (type-a,b). *REIC* type-a mRNA has shown to be a major transcript in various cancer cells, and its promoter activity was much stronger than that of type-b. In this study, we examined the methylation status of *REIC/Dkk-3* type-a in a broad range of human malignancies.
Methods We examined *REIC/Dkk-3* type-a methylation in breast cancers, non-small-cell lung cancers, gastric cancers, colorectal cancers, and malignant pleural mesotheliomas using a quantitative combined bisulfite restriction analysis assay and bisulfate sequencing. *REIC/Dkk-3* type-a and type-b expression was examined using reverse transcriptional PCR. The relationships between the methylation and clinicopathological factors were analyzed.
Results The rate of *REIC/Dkk-3* type-a methylation ranged from 26.2 to 50.0% in the various primary tumors that were examined. *REIC/Dkk-3* type-a methylation in breast cancer cells was significantly heavier than that in the other cell lines that we tested. *REIC/Dkk-3* type-a methylation was inversely correlated with *REIC/Dkk-3* type-a expression. There was a correlation between *REIC/Dkk-3* type-a and type-b mRNA expression. *REIC/Dkk-3* type-a expression was restored in MDA-MB-231 cells using 5-aza-2′-deoxycytidine treatment. We found that estrogen receptor–positive breast cancers were significantly more common among the methylated group than among the non-methylated group.
Conclusions *REIC/Dkk-3* type-a methylation was frequently detected in a broad range of cancers and appeared to play a key role in silencing *REIC/Dkk-3* type-a expression in these malignancies.

Keywords DNA methylation · *REIC/Dkk-3* · Breast cancer · Lung cancer · Mesothelioma

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Introduction

Accumulating evidence suggests that tumor progression is governed not only by genetic changes intrinsic to cancer cells but also by epigenetic changes. In cancer epigenetics, aberrant CpG methylation in the promoter region is a key mechanism for gene inactivation, resulting in tumorigenesis in human malignancies (Toyooka and Shimizu 2004).

The *REIC (reduced expression in immortalized cells)/Dkk-3(Dickkopf-3)* cDNA, which was expressed in human normal cells and was down-regulated in human immortalized cells and human tumor-derived cells, was identified using a representative difference analysis system (Tsuji et al. 2000). The amino acid sequence revealed that the *REIC* gene product was human *Dkk-3*, one of the Dkk family members. The Dkk family of secreted proteins consists

of four members, which share two conserved cysteine-rich domains (Glinka et al. 1998; Krupnik et al. 1999). Dkk-1, the best-characterized member of the Dkk family, functions as a Wnt antagonist or agonist by binding to and inhibiting or activating the Wnt coreceptor LRP6 (Bafico et al. 2001). Unlike Dkk-1, Dkk-2, and Dkk-4, however, REIC/Dkk-3 was recently shown to inhibit TCF-4 receptor activity in lung cancer cells (Yue et al. 2008). TCF-4 activates c-Myc and cyclin D1 through the Wnt/beta-catenin pathway and promotes tumor invasion and metastasis. Because *REIC/Dkk-3* is down-regulated in a variety of malignancies and the overexpression of *REIC/Dkk-3* suppresses cell growth, *REIC/Dkk-3* has been proposed to act as a tumor suppressor (Tsuji et al. 2001; Kurose et al. 2004). Hypermethylation and the down-regulation of *REIC/Dkk-3* were observed in a variety of malignancies including non-small-cell lung cancers (NSCLCs) (Kobayashi et al. 2002; Licchesi et al. 2008), gastrointestinal cancers (Maehata et al. 2008), renal clear cell carcinoma (Kurose et al. 2004), acute lymphoblastic leukemia (Roman-Gomez et al. 2004) and osteosarcomas (Hoang et al. 2004). We previously showed the therapeutic effect of REIC/Dkk-3 in prostate cancers (Abarzua et al. 2005; Edamura et al. 2007) and malignant pleural mesothelioma (MPM) (Kashiwakura et al. 2008). In addition, tumor suppression by REIC/Dkk-3 has also been confirmed in other malignant tumors (Hsieh et al. 2004; Hoang et al. 2004).

REIC/Dkk-3 mRNA has two isoforms (type-a,b; GenBank accession AB057804). Many papers have described the methylation status in the promoter of *REIC/Dkk-3* type-b (Licchesi et al. 2008; Maehata et al. 2008; Veeck et al. 2009). However, the promoter of *REIC/Dkk-3* type-a also seems to be important, since Kobayashi et al. (2002) (the group that first identified the *REIC/Dkk-3* in immortalized cells) have demonstrated that the promoter activity of *REIC/Dkk-3* type-a (major promoter) had an approximately 26-fold stronger effect than that of *REIC/Dkk-3* type-b (minor promoter) in a luciferase assay, and the major transcript was *REIC/Dkk-3* type-a in various cancer cells they tested. They suggested that hypermethylation of the major promoter (type-a) was a major mechanism for the down-regulation of *REIC* expression. They also suggested that the methylation of the minor promoter (type-b) was accompanied with that of major promoter (type-a) in most cases except four lung cancer cells that they tested. Regardless, those four lung cancer cells had type-b hypermethylation, *REIC/Dkk-3* type-b expression was detected in those four lung cancer cells. So they discussed the possibility that minor promoter (type-b) was utilized for the expression in a tissue-specific manner, as seen in dual promoter of APC gene.

In this study, we examined the DNA methylation of *REIC/Dkk-3* type-a in various kinds of cancers by quantitative combined bisulfite restriction analysis (qCOBRA) and investigated the correlation between the *REIC/Dkk-3* type-a methylation and *REIC/Dkk-3* type-a expression. The qCOBRA assay can provide more reliable results because the conventional methylation-sensitive restriction enzyme assay that Kobayashi et al. (2002) performed was recently known to be prone to false-positive results due to spurious incomplete digestion (Xiong and Laird 1997). We also analyzed the correlation between *REIC/Dkk-3* type-a and type-b expression in various cancer cell lines. Furthermore, we examined the correlation between *REIC/Dkk-3* type-a methylation and the clinicopathological features of primary tumors.

Materials and methods

Clinical samples and cell culture

Surgically resected specimens of 37 primary breast cancers, 42 primary NSCLCs, 21 primary gastric cancers, 20 primary colon cancers, and 7 MPMs were obtained from Okayama University Hospital (Okayama, Japan), 6 MPMs were obtained from Okayama Rousai Hospital (Okayama, Japan), 5 MPMs were obtained from National Sanyo Hospital (Yamaguchi, Japan), and 27 MPMs were obtained from Karmanos Cancer Center (MI). Ten corresponding non-malignant breast tissues and 10 non-malignant lung tissues were also examined. All tissues were frozen with the liquid nitrogen immediately after surgery and were stored at −80°C until extraction of DNA. Institutional Review Board permission and informed consent were obtained for all cases.

Seven breast cancer cell lines (HCC70, HCC1599, HCC1806, MDA-MB-231, MDA-MB-361, MCF7, and ZR75-1), 11 lung cancer cell lines (NCI-H23, NCI-H44, NCI-H125, NCI-H157, NCI-H1299, NCI-H1819, NCI-H1963, NCI-H1975, NCI-H2009, NCI-H358, and A549), 4 MPM cell lines (NCI-H2052, NCI-H2373, NCI-H2452, and NCI-H290), and 6 prostate cancer cell lines (PC3, LNCap-FGC, Du145, Caki-1, Caki-2, and KPK) were examined in this study. MCF7, ZR-75-1, MDA-MB-231, and MDA-MB-361 were obtained from Cell Resource Center for Biomedical Research Institute of Development Aging and Cancer Tohoku University (Miyagi, Japan). Seven cell lines (HCC70, HCC1599, HCC1806, H2052, H2373, H290, and H2452) were kind gifts from Adi F. Gazdar (Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX). Six cell lines (PC3, LNCap-FGC, Du145, Caki-1, Caki-2, and KPK) were kind gifts from the department of urology (Okayama University, Okayama, Japan). The other cell lines were obtained from American Type Culture Collection (Manassas, VA). The cells

were maintained in RPMI-1640 medium (Sigma Chemical Co., Saint Louis, MO) supplemented with 10% FBS and were incubated in 5% CO_2.

DNA extraction and DNA methylation modification

Genomic DNA was extracted from the surgically resected frozen samples and cultured cells by digestion with SDS/proteinase K followed by phenol/chloroform (1:1) extraction and ethanol precipitation. Two micrograms of each DNA was treated with EZ DNA Methylation Kit (ZYMO RESEARCH, Orange, CA), following the manufacturer's instructions, and was stored at −20°C until use.

Quantitative COBRA assay

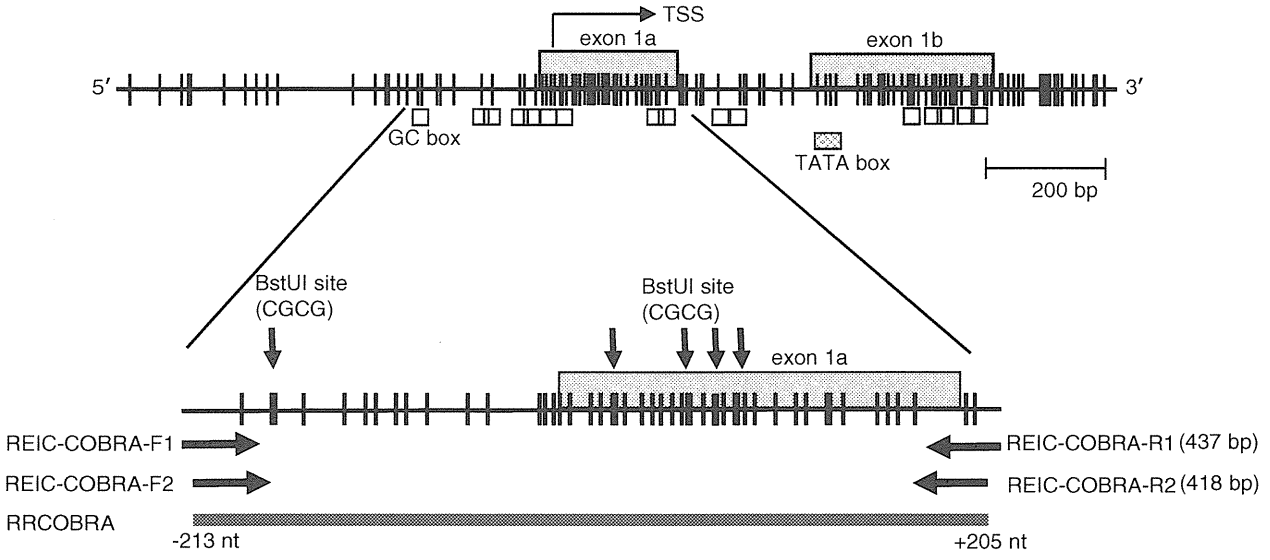
Nested PCR was carried out using bisulfite-treated DNA followed by the restriction enzyme digestion. First-round touchdown PCR was performed under the following conditions: 95°C for 12 min, 40 cycles of 94°C for 45 s, annealing temperature between 58 and 56°C for 1 min, 72°C for 3 min, followed by final extension step at 72°C for 7 min in a 25-μl reaction mixture containing 67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mm $MgCl_2$, 10 mM β-mercaptoethanol, 1.25 mM of each deoxynucleotide triphosphate (dNTP) mixture, 0.5 μM of each primer, 0.5 unit of HotStar Taq DNA Polymerase (Qiagen, Valencia, CA), and 100 ng of bisulfite-treated DNA. Second-round touchdown PCR was performed using 0.4 μl of the first-round PCR products as a template under same condition, but 47 cycles. Universal methylated DNA and universal unmethylated DNA were used for positive control and negative control, respectively. The location of the CpG dinucleotides in the exon1 and in the 5′-flanking region of *REIC/Dkk-3* is shown in Fig. 1. Primers were designed using Primer Express software ver.1.0 in the promoter region of *REIC/Dkk-3* type-a. Primers for the first-round PCR were *REIC*-COBRA-F1 5′-TGGGTTGTTGTAAGTTTGAAGGT-3′ and *REIC*-COBRA-R1 5′-CTCACCCACCCCRACTAAAC-3′. Primers for the second-round PCR were as follows: *REIC*-COBRA-F2 5′-TGAAGGTTAGATAAGAYGGGTTTAGG-3′ and *REIC*-COBRA-R2 5′-ACCCACCCCRACTAAACCRAAT-3′. These primers were designed to ensure amplification of both methylated and unmethylated forms. Two microliters of second PCR products were digested with 3 units of BstUI (whose restriction site is CGCG) for the restriction fragment length polymorphism analysis. The amplicon of second PCR was named RRCOBRA (Region for REIC-COBRA), and the 5 restriction sites of BstUI are shown in Fig. 1. The digested PCR products were visualized on 3% agarose gels stained with ethidium bromide. The percentages of digested band were analyzed by NIH ImageJ 1.37 V software (http://rsb.info.nih.gov/ij) as described previously (Xiong and Laird 1997). We performed linear regression analysis of qCOBRA with nested PCR using serial dilution to examine whether qCOBRA with nested PCR really reflected % methylation. We diluted unmethylated DNA amplicon with methylated amplicon to make serial dilution (% methylated DNA; 0, 10, 20, 30, 50, 70, 80, 90, 100%) and performed qCOBRA, as described above.

Bisulfited DNA sequencing analysis

RRCOBRA was cloned into pCR2.1-TOPO Vector using TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA) following manufacturer's instructions. To determine the methylation status in the promoter lesion of *REIC/Dkk-3* gene, five breast cancer cell lines (MCF-7, MDA-MB-231, ZR75-1, HCC1806, and HCC1599) and a lung cancer cell line (H1299) were examined. Seven individual clones from each cell line were sequenced using the dGTP BigDye terminator v3.1 Cycle Sequencing Kit with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

RNA extraction and reverse transcriptional (RT)-PCR

Total RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instruction. Oligo(dT)-primed cDNA was synthesized using Super-Script II (Qiagen, Valencia, CA) with DNase treatment. RT-PCR was carried out in 20 μl of reaction mixture with 1xPCR buffer, 200 μM of dNTP, 0.3 μM of each primer, 0.5 units of HotStarTag DNA Polymerase, and 100 ng of cDNA. A touchdown PCR was performed for *REIC/Dkk-3* type-a and type-b under the following conditions: 95°C for 12 min, 35 cycles of 94°C for 30 s, annealing temperature between 62 and 58°C for 1 min, 72°C for 3 min, followed by final extension step at 72°C for 7 min. As an internal control, RT-PCR for *GAPDH* was carried out under the following conditions: 95°C for 12 min, 35 cycles of 94°C for 45 s, 55°C for 90 s, 72°C for 90 s, followed by final extension step at 72°C for 7 min. The primers for *REIC/Dkk-3* type-a expression were *REIC* (a)-F 5′-GGGAGCGAGCAGATCCAGT-3′ (exon1a) and *REIC*(a)-R 5′-TTTGTCCAGTCTGGTTGTTGGT-3′ (exon3). The primers for *REIC/Dkk-3* type-b expression were *REIC*(b)-F 5′-TGGGAGCTATTAGCGTAGAGGA T-3′ (exon1b) and *REIC*(b)-R 5′-CATTGTGATAGCTGG GAGGTAAG-3′ (exon3). The PCR products were visualized on 2% agarose gels stained with ethidium bromide. The bands were analyzed using NIH ImageJ 1.37 V software. The expression ratio in each cell line was defined as the ratio of particular sample when compared to those of H1299. To confirm the responsibility of DNA methylation for *REIC/Dkk-3* silencing, we treated heavily methylated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exon 1a
REIC-COBRA-F1
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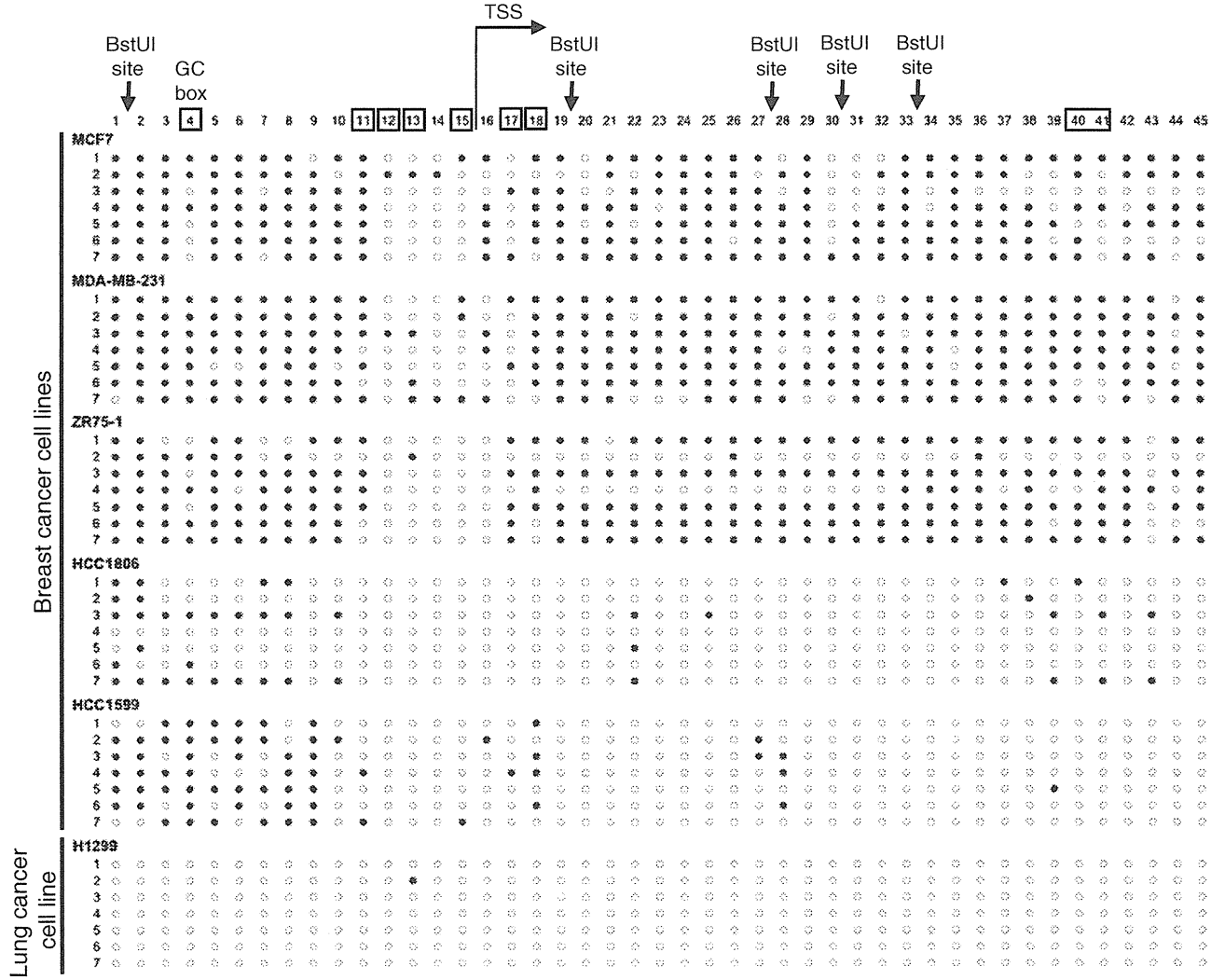
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MCF7
MDA-MB-231
ZR75-1
HCC1806
HCC1599
H1299
Breast cancer cell lines
Lung cancer cell line