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Molecular analysis of metaplastic breast carcinoma: high *EGFR* copy number via aneusomy

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Abstract

Metaplastic breast carcinoma, a rare tumor composed of adenocarcinomatous and nonglandular growth patterns, is characterized by a propensity for distant metastases and resistance to standard anticancer therapies. We sought confirmation that this tumor is a basal-like breast cancer, expressing epidermal growth factor receptor (EGFR) and stem cell factor receptor (KIT). *EGFR* activating mutations and high copy number (associated with response to tyrosine kinase inhibitor gefitinib) and *KIT* activating mutations (associated with imatinib sensitivity) were then investigated. Seventy-seven metaplastic cases were identified (1976-2006); 38 with tumor blocks available underwent pathologic confirmation before EGFR and KIT immunohistochemical analyses. A tissue microarray of malignant glandular and metaplastic elements was constructed and analyzed immunohistochemically for cytokeratin 5/6, estrogen receptor, progesterone receptor, and p63, and by fluorescence *in situ* hybridization for *EGFR* and *HER-2/neu*. DNA isolated from individual elements was assessed for *EGFR* and *KIT* activating mutations. All assessable cases were negative for estrogen receptor, progesterone receptor, and (except one) HER2. The majority were positive for cytokeratin 5/6 (58%), p63 (59%), and EGFR overexpression (66%); 24% were KIT positive. No *EGFR* or *KIT* activating mutations were present; 26% of the primary metaplastic breast carcinomas were fluorescence *in situ* hybridization-positive, displaying high *EGFR* copy number secondary to aneusomy (22%) and amplification (4%). We report here that metaplastic breast carcinoma is a basal-like breast cancer lacking *EGFR* and *KIT* activating mutations but exhibiting high *EGFR* copy number (primarily via aneusomy), suggesting that EGFR tyrosine kinase inhibitors should be evaluated in this molecular subset of breast carcinomas. [Mol Cancer Ther 2008;7(4):944–51]

Introduction

Metaplastic breast carcinomas are a heterogeneous group of tumors in which the adenocarcinomatous element is admixed with one or more squamous, spindle, chondroid, or osseous neoplastic components (1, 2). Metaplastic breast cancer is rare, accounting for <5% of all breast malignancies. An earlier Mayo Clinic study indicated that although more frequently node-negative at presentation, metaplastic breast carcinoma is more aggressive than breast adenocarcinoma without metaplasia, having an increased risk of locally recurrent and metastatic disease (3). Furthermore, regimens conventionally employed for metastatic breast cancer appear to be less effective for metastatic metaplastic breast carcinoma in this series.

Comparison of gene expression profiles of breast carcinomas (4–6) has validated the traditional classification of these molecularly diverse tumors into two broad groups, those positive or those negative for estrogen receptor (ER) expression. ER-negative tumors have been subdivided into normal breast-like, basal epithelial-like, and HER2 (ErbB2) overexpressing subclasses (4). The basal epithelial-like subgroup of breast carcinomas is characteristically negative for ER, progesterone receptor (PR), and HER2-overexpression (that is, ''triple negative'') but positive for EGFR (epidermal growth factor receptor 1, ErbB1, HER1), KIT (stem cell factor receptor; mast cell growth factor receptor), cytokeratin 5/6 (CK 5/6), and p63 (7, 8). Clinically, the basal-like breast tumor subtype is associated with a poorer prognosis in terms of relapse-free survival and overall survival (5, 6, 9–11). Early literature reports indicate that the vast majority of metaplastic breast carcinomas, the subject of this report, are also negative for ER, PR, and HER2-overexpression as well as positive for EGFR, CK 5/6, and p63 expression (12–14), suggesting that these tumors may exhibit characteristics associated with basal-like breast carcinomas (14).

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Note: J.A. Gilbert and M.P. Goetz contributed equally to this work.

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Because metaplastic breast carcinomas are characteristically negative for ER and HER2 and because these tumors are often unresponsive to conventional chemotherapeutic regimens (3), treatment options are limited and new drug therapies are urgently needed. *EGFR* mutations in exons 18, 19, and 21 are associated with response to the tyrosine kinase inhibitor gefitinib in non-small cell lung cancer (NSCLC; refs. 15, 16). A recent report showed that although EGFR was overexpressed in 68% of metaplastic breast carcinomas, *EGFR* activating mutations in exons 18 to 21 were not present (17). Additionally, the above study reported various levels of *EGFR* amplification measured by chromogenic *in situ* hybridization (CISH) in 23% of metaplastic tumors. Because high *EGFR* copy number detected by fluorescence *in situ* hybridization (FISH) (either via gene amplification or high polysomy/aneusomy in which the increased number of *EGFR* copies is detected with a balanced increase in the number of chromosome 7 copies) is associated with gefitinib response in lung cancer (18, 19), FISH-positivity in metaplastic breast carcinoma may be a useful marker for identifying patients who may benefit from EGFR inhibitors but has never been analyzed. Analogously to *EGFR*, activating mutations in *KIT* (*C-Kit*, *CD117*) exons 9, 11, 13, and 17 are associated with response of gastrointestinal stromal tumors to the tyrosine kinase inhibitor imatinib (20), but their presence in metaplastic breast carcinoma is unknown. This study was conducted to examine the basal immunohistochemical profile, activating mutations in *EGFR* and *KIT*, and *EGFR* and *HER-2/neu* copy numbers by FISH in a panel of metaplastic breast carcinomas.

Materials and Methods

Patient Samples

The Mayo Clinic medical index was queried from 1976 to 2006 with the following terms: ''metaplastic breast cancer,'' ''spindle cell cancer,'' ''squamous cell cancer,'' ''cancer with sarcomatoid features,'' ''chondroid metaplasia,'' ''bony or osseous metaplasia,'' ''breast cancer-chondroid metaplasia,'' ''breast cancer-sarcomatous metaplasia,'' ''breast cancer-spindle cell metaplasia,'' or ''breast cancer-squamous metaplasia.'' A total of 77 patients were identified with one of these diagnoses. Of these, pathology slides were available for review in 49 patients. Before inclusion of a case in this study, an H&E slide from each associated block was reviewed by a pathologist to confirm the diagnosis of metaplastic breast carcinoma. Of these 49 cases, the diagnosis of metaplastic carcinoma was confirmed in 45 patients by the study breast pathologist (C.A.R.). Formalin-fixed, paraffin-embedded tumor blocks were available in 38 of 45. These 38 cases comprise the study cohort. The present study was reviewed and approved by the Mayo Clinic Institutional Review Board.

Immunohistochemical Analysis

Sections (5 μm) of the tumor blocks were analyzed by immunohistochemistry for KIT and EGFR. Immunohistochemical staining for EGFR was scored based on intensity from 0 to 3+ as per manufacturer's guidelines, with 0 indicating absence of staining, and 1+, 2+, and 3+ representing weak, moderate, and strong staining intensity, respectively. EGFR overexpression was defined as a score of either 2+ or 3+. Figure 1 illustrates a representative case of metaplastic breast carcinoma for each level of EGFR

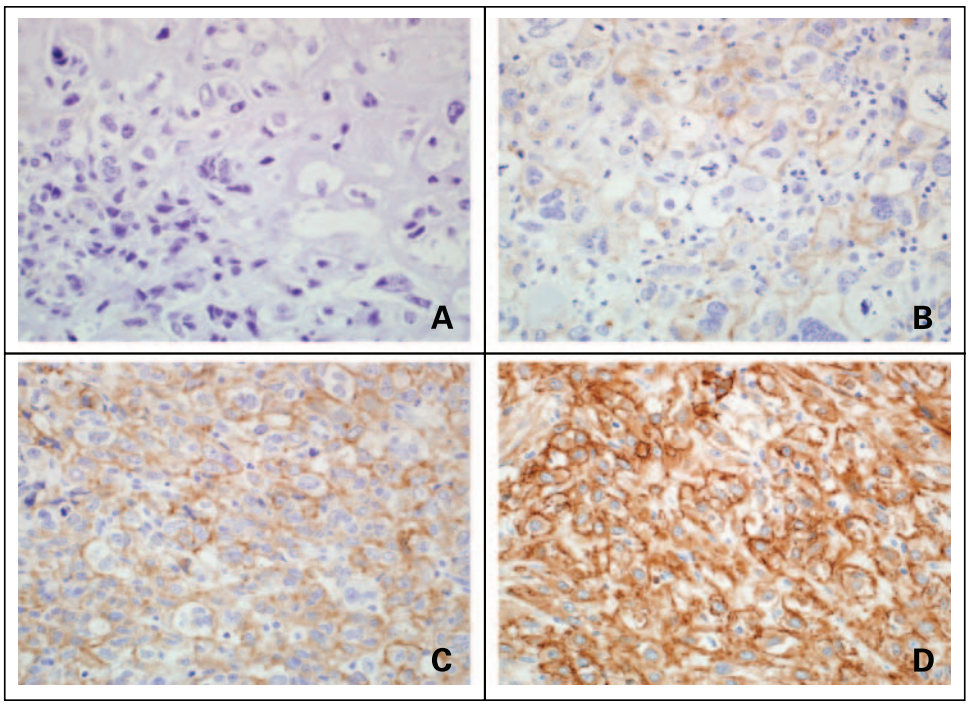

Figure 1. Immunohistochemical analysis of EGFR in metaplastic breast carcinomas. Individual specimens with staining intensity scores of (**A**) 0, (**B**) 1+, (**C**) 2+, and (**D**) 3+, with EGFR overexpression being defined as an intensity of 2+ or 3+.

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immunohistochemical staining. KIT staining was scored as either negative or positive. Tissue microarray sections (5 μm) were stained for p63, CK 5/6, ER, and PR, with staining scored as negative or positive. If immunohistochemistry scores of any biomarker differed among a patient's tumor sections, the greatest intensity score was reported (12 patients). All immunohistochemistry antibodies and methods are listed in Supplementary Table S1.[8]

EGFR and *KIT* Mutational Analysis

Histologic review of H&E-stained sections from the 56 tumor blocks accessible for the 38 metaplastic breast carcinoma cases (1-3 blocks per patient) identified malignant glandular, squamous, spindle cell, chondroid, and osseous components. Each individual element was circled on an H&E slide, and the corresponding area of tissue was removed from three unstained (10 μm) sections of the associated tumor block. DNA was isolated from each of these individual elements with the QIAamp DNA Mini Kit (Qiagen) such that a total of 73 unique aliquots of DNA were collected for mutational analysis (that is, DNA samples from 13 glandular, 11 squamous, 38 spindle cell, 1 osseous, and 10 chondroid components).

EGFR and *KIT* exons of interest were amplified by use of PCR with a modification of the technique for *EGFR* mutational analysis of Gilbert et al. (21). PCR primers for the selected exons were designed to hybridize at locations that resulted in the production of amplicons containing predictive mutation sites as well as ≤181 bp of exon sequence (with the exception of the *KIT* exon 11 amplicon, which contained 206 bp of exon sequence). All primer sequences and annealing temperatures are listed in Supplementary Table S2.[8] Amplifications were done with iQ Supermix (Bio-Rad) with 1.25 units (final) iTaq DNA Polymerase (Bio-Rad). All PCR amplifications were done in a Perkin-Elmer model 9700 thermal cycler.

Amplicons were sequenced on both strands in the Mayo DNA Sequencing Facility using the universal M13 forward and reverse sequences as primers (except for *KIT* exon 13, for which the forward PCR primer was employed instead of the M13 forward sequence). ABI BigDye Terminator sequencing chemistry was employed with an ABI 3730 DNA sequencer, and sequencing chromatograms were analyzed using Sequencher 4.5 (Gene Codes). NT_033968.5 and NM_005228.3 were the Genbank accession numbers for the *EGFR* reference sequences used in these studies, whereas NT_022853.14 and U63834 were the reference sequences employed for *KIT*.

Genomic DNA controls containing *EGFR* mutations encoding L858R in exon 21, delE746-A750 in exon 19, and delL747-P753insS in exon 19 were kind gifts of Drs. Daphne Bell and Daniel Haber (Massachusetts General Hospital). HMC-1 cells with *KIT* mutations encoding V560G in exon 11 and D816V in exon 17 were generously provided by Dr. Joseph Butterfield (Mayo Clinic Rochester).

[8] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Table 1. Characteristics of 30 primary metaplastic breast carcinoma cases

Median age (range)	61 y (34-90)
Year of surgery	
1976-1979	10.0%
1980-1989	6.7%
1990-1999	33.3%
2000-2005	50.0%
Prior history of cancer	20.0%
Maximum tumor dimension (cm)	
<2	20%
2-5	60%
>5	20%
No. positive nodes	
Not examined	13.3%
0	63.3%
1-3	16.7%
4-9	3.3%
10+	3.3%
Nottingham grade	
1	3.3%
2	23.3%
3	73.3%
Tumor histology	
Spindle cell	43.3%
Chondroid	3.3%
Mixture of glandular and metaplastic elements	
Glandular and squamous	3.3%
Glandular and spindle cell	13.3%
Glandular and chondroid	6.7%
Glandular with squamous and spindle cell	10.0%
Glandular with squamous and chondroid	3.3%
Glandular with spindle cell and chondroid	3.3%
Mixture of metaplastic elements	
Squamous and spindle cell	6.7%
Spindle cell and chondroid	3.3%
Spindle cell, chondroid, and osseous	3.3%
Presence of metastatic disease	3.3%

Tissue Microarray Construction

A tissue microarray was constructed with a Beecher ATA-27 automated arrayer from the 73 individual malignant glandular and metaplastic elements identified previously in the tissue blocks of the 38 metaplastic breast carcinoma cases. Each of these individual elements was circled on an H&E slide, and triplicate 0.6-mm cores were removed from the corresponding area of tissue in the associated tumor block and placed into a single recipient paraffin block.

Gene Copy Number Analysis

Tissue microarray sections (5 μm) were analyzed by FISH for *EGFR* and *HER-2/neu* copy number. FISH for *HER-2/neu* (*ERBB2*) was done with the PathVysion HER2 DNA Probe Kit (Vysis; ref. 22). FISH for *EGFR* was done with the LSI EGFR/CEP 7 Probe (Vysis) as per manufacturer's instructions. Thirty nuclei were scored per sample, and the number of *HER-2/neu* or *EGFR* (red) signals and chromosome 17 centromere or chromosome 7 centromere (green) signals, respectively, were recorded. A ratio of *HER-2/neu*:chromosome

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17 centromere or *EGFR*:chromosome 7 centromere of 0.8 to 1.30 was defined as normal, a ratio of <0.8 was interpreted as gene deletion, a ratio of 1.30 to 2.0 was defined as gene duplication, and a ratio ≥2.0 was interpreted as gene amplification. *EGFR* FISH-positive samples were those showing amplification or high aneusomy with ≥40% of cells having ≥4 copies of *EGFR*, where aneusomy was defined as a normal *EGFR*:chromosome 7 centromere ratio with >30% of cells having ≥3 chromosome 7 centromere signals (that is, a balanced gain in *EGFR* and chromosome 7 centromere copy numbers). If any element within a patient's tumor was FISH-positive, the tumor was reported to be FISH-positive (3 patients).

Results

Clinical Characteristics of Metaplastic Breast Carcinoma Cases

Seventy-seven metaplastic cases were identified between 1976 and 2006 at Mayo Clinic. Of the 49 cases with pathology slides available, a diagnosis of metaplastic breast carcinoma was confirmed in 45 patients, 38 that had accessible tumor blocks. This study of 38 patients with metaplastic breast carcinoma includes 30 (cohort A) who underwent excision or reexcision of primary disease and 8 (cohort B) who underwent either excision of disease following neoadjuvant chemotherapy (2 patients) or excision of recurrent/metastatic disease (6 patients). The

Table 2. Individual immunohistochemical and FISH results for metaplastic breast carcinoma cases (*n* = 38)

Case no.	Tumor histology	EGFR*	EGFR FISH†	KIT*	CK 5/6‡	p63‡	ER‡	PR‡	HER2 FISH†
1	Spindle cell	1+	-	-	-	-	-	-	-
2	Chondroid	3+	-	+	+	ND	ND	-	-
3	Glandular and spindle cell	3+	+	-	+	+	-	-	-
4	Spindle cell	2+	-	-	-	-	-	-	-
5	Spindle cell	0	-	-	+	-	-	-	-
6	Spindle cell, chondroid, and osseous	1+	-	-	-	-	-	-	-
7	Glandular with squamous and spindle cell	3+	-	-	+	+	-	-	-
8	Glandular and spindle cell	3+	+	-	-	+	-	-	-
9	Glandular with squamous and spindle cell	2+	ND§	-	ND	ND	ND	-	-
10	Squamous	1+	-	-	+	+	-	-	-
11	Spindle cell	1+	-	-	+	-	-	-	-
12	Glandular with squamous and spindle cell	3+	-	+	+	+	-	-	-
13	Glandular and spindle cell	2+	-	-	+	+	-	-	-
14	Glandular and squamous	3+	-	+	+	+	-	-	-
15	Glandular with squamous and spindle cell	3+	-	-	+	+	-	-	-
16	Squamous and spindle cell	1+	+	-	+	+	-	-	-
17	Spindle cell	2+	-	-	-	+	-	-	-
18	Squamous and spindle cell	3+	-	-	+	+	-	-	-
19	Glandular and spindle cell	1+	-	+	-	-	-	-	-
20	Spindle cell	1+	-	-	-	-	-	-	-
21	Spindle cell	3+	-	-	-	-	-	-	-
22	Spindle cell	3+	-	-	-	+	-	-	-
23	Spindle cell and chondroid	2+	+	+	+	-	-	-	-
24	Glandular and chondroid	1+	-	+	-	-	-	-	-
25	Spindle cell	0	ND	-	ND	ND	ND	ND	ND
26	Spindle cell	3+	-	-	+	+	-	-	-
27	Glandular with squamous and spindle cell	3+	+	-	+	+	-	-	-
28	Glandular with spindle cell and chondroid	1+	-	+	+	+	-	-	-
29	Glandular and spindle cell	3+	ND	-	+	ND	ND	ND	-
30	Glandular and spindle cell	2+	-	+	+	+	-	-	+
31	Glandular and chondroid	2+	-	+	+	+	-	-	-
32	Spindle cell	3+	+	-	+	+	-	-	-
33	Glandular with squamous and chondroid	2+	+	-	+	-	-	-	-
34	Spindle cell	3+	+	-	-	+	-	-	-
35	Spindle cell	3+	-	-	-	+	-	-	-
36	Spindle cell	3+	-	-	-	-	-	-	-
37	Spindle cell	1+	-	-	-	-	-	-	-
38	Spindle cell	1+	-	-	-	-	-	-	-

*As determined by immunohistochemistry of sections from tumor blocks.
†As determined by FISH of tissue microarray sections. ''EGFR FISH,'' determination of FISH-positivity (aneusomy with ≥40% of cells having ≥4 copies of *EGFR* or *EGFR* amplification); ''HER2 FISH,'' determination of *HER-2/neu* amplification.
‡As determined by immunohistochemistry of tissue microarray sections.
§ND, not determined due to tissue dropout in the tissue microarray.

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Table 3. Subgroup analysis of biomarker expression in metaplastic breast carcinomas (n = 38 cases)

Tumor histology	EGFR overexpression*		*EGFR* FISH-positive†		KIT expression*		CK 5/6 expression‡		p63 expression‡	
	Primary	Other	Primary	Other	Primary	Other	Primary	Other	Primary	Other
Squamous		0/1		0/1		0/1		1/1		1/1
Spindle cell	8/13	1/3	2/12§	0/3	0/13	0/3	2/12§	2/3	5/12§	1/3
Chondroid	1/1		0/1		1/1		1/1		§	
Mixture of glandular and metaplastic elements										
Glandular and squamous	1/1		0/1		1/1		1/1		1/1	
Glandular and spindle cell	4/4	1/2	1/3§	1/2	1/4	1/2	3/4	1/2	3/3§	1/2
Glandular and chondroid	1/2		0/2		2/2		1/2		1/2	
Glandular with squamous and spindle cell	3/3	2/2	1/2§	0/2	0/3	1/2	2/2§	2/2	2/2§	2/2
Glandular with squamous and chondroid	1/1		1/1		0/1		1/1		0/1	
Glandular with spindle cell and chondroid	0/1		0/1		1/1		1/1		1/1	
Mixture of metaplastic elements										
Squamous and spindle cell	1/2		1/2		0/2		2/2		2/2	
Spindle cell and chondroid	1/1		1/1		1/1		1/1		0/1	
Spindle cell, chondroid, and osseous	0/1		0/1		0/1		0/1		0/1	

NOTE: Value represents (number positive) / (total number of patients per tumor subtype). Results were separated according to case type, either primary (cohort A) or other (cohort B). All assessable tumors were negative for ER, PR, and (except one) *HER-2/neu* amplification.
*As determined by immunohistochemistry of sections from tumor blocks. ''EGFR overexpression,'' intensity of 2+ or 3+.
†Aneusomy with ≥40% of cells having ≥4 copies of *EGFR* or (for one tumor) *EGFR* amplification, as determined by FISH analysis of tissue microarray sections.
‡As determined by immunohistochemistry of tissue microarray sections.
§Results for some tumors not available due to tissue dropout in tissue microarray.

median age at surgery of cohort A was 61 years (range, 34-90); six of these patients had been previously diagnosed with another cancer. At presentation, cohort A tumors tended to be 2 to 5 cm in size (60%), to be Nottingham grade 3 (73%), to have a spindle cell component (83%), and to be node-negative (63%; Table 1). Cohort B were in various stages of their disease course and had received a variety of treatments before surgery for this instance of metaplastic breast cancer.

Immunohistochemistry

Of 38 metaplastic breast carcinoma cases, all those assessable were negative by immunohistochemistry for ER and PR, and by FISH for *HER-2/neu* amplification (except for one primary). By immunohistochemistry, EGFR was overexpressed in 70% of cohort A, whereas 54% were CK 5/6 positive, 58% were p63 positive, and 23% were KIT positive. Of cohort B, ≥50% exhibited EGFR overexpression (4 of 8), CK 5/6 positivity (6 of 8), and p63 positivity (5 of 8). A summary of the immunohistochemical and FISH results for the 38 individual metaplastic breast carcinoma cases is shown in Table 2, and a subgroup analysis of biomarker expression for all 38 cases is provided in Table 3.

EGFR and *KIT* Mutational Analysis

EGFR exons 18, 19, and 21 and *KIT* exons 9, 11, 13, and 17 were assessed for mutations in 73 DNA samples isolated from malignant glandular and metaplastic components of 38 metaplastic breast carcinoma tumors. Sequence was obtained for all *EGFR* and *KIT* exons in all 73 DNA samples from the 38 cases, except for one sample (the spindle cell component of a tumor composed of spindle cell, chondroid, and osseous elements) in which no sequence for any of the *EGFR* or *KIT* exons could be obtained and one sample (the osseous component of the tumor composed of spindle cell, chondroid, and osseous elements) in which neither *EGFR* exon 21 nor *KIT* exon 11 could be sequenced. No *EGFR* or *KIT* activating mutations were found among these cases.

EGFR Copy Number Analysis

Of the 27 (of 30) primary metaplastic breast carcinoma cases assessable in the tissue microarray for *EGFR* copy number by FISH analysis, 7 (26%) displayed high *EGFR* copy number. One of the cohort B cases also showed high *EGFR* copy number. Of these eight FISH-positive metaplastic breast carcinomas, one (a primary) showed *EGFR* amplification and seven displayed aneusomy with ≥4 *EGFR* copies in ≥40% of cells—most frequently in the spindle cell component (75%). Figure 2 illustrates examples of the FISH-positive metaplastic breast tumors. Summaries of the *EGFR* FISH results for all of the metaplastic breast carcinomas by case and by cohort are provided in Tables 2 and 3, respectively, whereas Table 4 presents the characteristics of the individual FISH-positive metaplastic breast tumors.

Discussion

This molecular study of metaplastic breast carcinomas confirmed previous findings that metaplastic tumors exhibit many characteristics typical of basal-like breast carcinomas, that is, ER/PR/HER2-negativity, p63 and CK 5/6 positivity, EGFR overexpression, and (approximately

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one-quarter) KIT positivity (7, 14). Additionally, we confirm the findings of Reis-Filho et al. (17) that metaplastic carcinomas do not harbor activating mutations in *EGFR*. We are, however, the first to show the absence of activating mutations in *KIT*. Most importantly, our demonstration that the malignant histologic elements (predominantly spindle cell) displayed high *EGFR* copy number (primarily via aneusomy) suggests that EGFR inhibitors should be investigated as a potential therapeutic agent for this subtype of breast cancer.

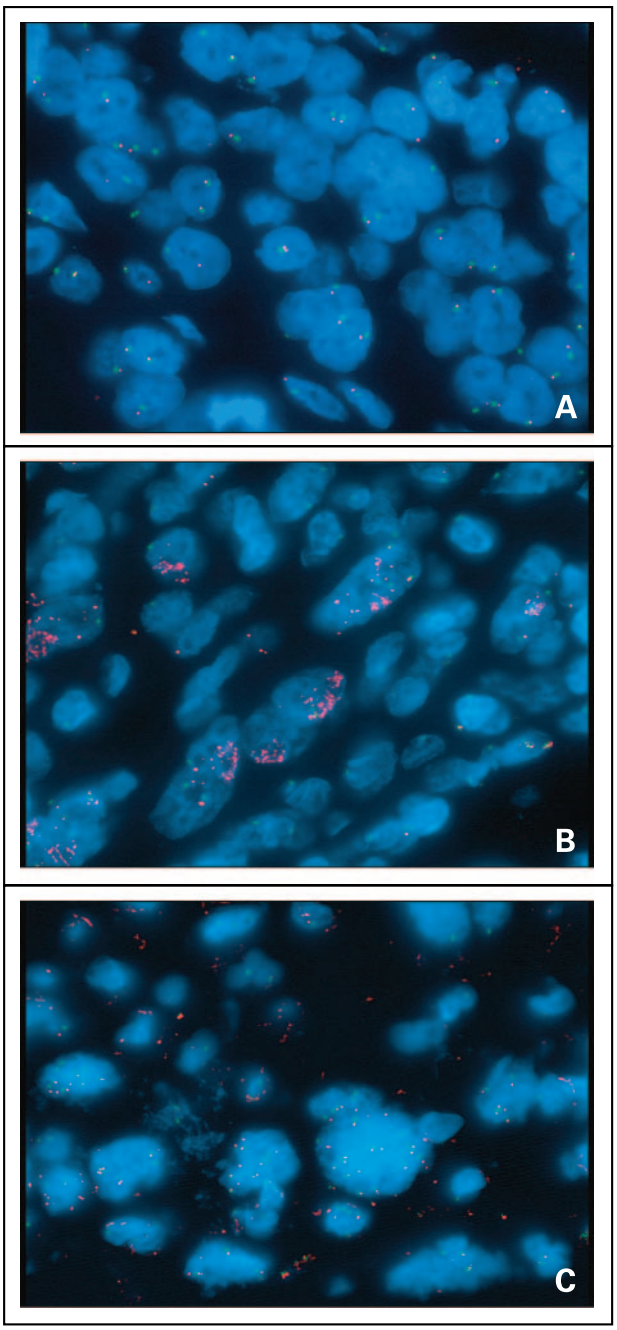

Figure 2. FISH analysis of *EGFR* copy number in metaplastic breast carcinomas. *Red, EGFR* signal; *green,* chromosome 7 signal. Individual specimens with (**A**) normal *EGFR* copy number, (**B**) amplification of *EGFR*, and (**C**) aneusomy with ≥40% of cells having ≥4 copies of *EGFR*.

The association of *EGFR* activating mutations with responsiveness to the tyrosine kinase inhibitor gefitinib observed in NSCLC (15, 16) has been the subject of numerous studies since the first publication in 2004. A recent review summarized mutational analyses of *EGFR* exons 18 to 21 in a total of 3,000 NSCLC cases (23); a retrospective comparison of mutational status with gefitinib response in 288 of these cases did not provide a perfect correlation although it did indicate that the majority of gefitinib-responsive NSCLC tumors harbored activating *EGFR* mutations. Subsequently, high *EGFR* gene copy number by FISH analysis has been associated with response to gefitinib in NSCLC (18) and bronchioloalveolar carcinoma subtypes (19). Specifically, *EGFR* FISH-positivity in tumors [defined as high balanced polysomy with ≥4 *EGFR* copies in ≥40% of cells or *EGFR* amplification (gene:chromosome ≥2 per cell, gene clusters, or ≥15 gene copies per cell in ≥10% of cells)] is associated with a higher gefitinib response rate in NSCLC (18). In bronchioloalveolar carcinoma subtypes of NSCLC, gefitinib responsiveness is also associated with *EGFR* FISH-positivity [high balanced polysomy with ≥4 *EGFR* copies in ≥40% of cells or *EGFR* amplification (gene:chromosome ≥2 per cell, gene clusters, or ≥15 gene copies per cell in ≥10% of cells; ref. 19)]. More recently, a prospective study of gefitinib in 42 NSCLC patients (24) reported objective responses in 48% of participants and confirmation of *EGFR* amplification and high polysomy as predictors of gefitinib responsiveness. Further, an association between gefitinib response and *EGFR* FISH-positivity in the absence of *EGFR* activating mutations was seen in a recent NSCLC study (25) in which 40% of patients lacking *EGFR* mutations exhibited FISH-positivity; notably, of the 21 nonmutants with high *EGFR* copy number, 24% responded to gefitinib treatment. Although the prognostic role of classical *EGFR* mutations and/or increased *EGFR* copy number requires further clarification, these factors are nonetheless considered predictive for tumor response to gefitinib (see ref. 26).

We report here that no *EGFR* activating mutations were found in these 38 metaplastic breast carcinoma cases. Our findings are comprehensive, because we sequenced DNA extracted from each of the malignant elements of metaplastic breast carcinoma (squamous, chondroid, adenocarcinomatous, osseous, and spindle). In addition, employing *EGFR* FISH criteria associated with gefitinib response in NSCLC (18, 19), *EGFR* FISH-positivity (amplification with gene:chromosome ≥2 per cell, or aneusomy with ≥4 *EGFR* copies in ≥40% of cells) was found in almost one-quarter of the assessable metaplastic breast carcinomas in this study (in 26% of the primary metaplastic breast carcinoma cases of cohort A). By comparison, one metaplastic breast carcinoma (spindle cell with focal squamous differentiation) included in a study of breast cancers by Bhargava et al. (27) showed high-level *EGFR*

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Table 4. Characteristics of FISH-positive metaplastic breast carcinomas

Case no.	Tumor Histology	EGFR*	*EGFR* FISH†	KIT*	CK 5/6‡	p63‡	ER‡	PR‡	HER2 FISH†
32	Spindle cell	3+	Aneusomy	-	+	+	-	-	-
34	Spindle cell	3+	Amplification	-	-	+	-	-	-
8	Glandular and spindle cell	3+	Aneusomy	-	-	+	-	-	-
3§	Glandular and spindle cell	3+	Aneusomy	-	+	+	-	-	-
27	Glandular with squamous and spindle cell	3+	Aneusomy	-	+	+	-	-	-
33	Glandular with squamous and chondroid	2+	Aneusomy	-	+	-	-	-	-
16	Squamous and spindle cell	1+	Aneusomy	-	+	+	-	-	-
23	Spindle cell and chondroid	2+	Aneusomy	+	+	-	-	-	-

NOTE: No FISH-positive tumors had *EGFR* (exons 18, 19, and 21) or *KIT* (exons 9, 11, 13, and 17) activating mutations.
*As determined by immunohistochemistry of sections from tumor blocks. EGFR staining was scored based on intensity from 0 to 3+, with overexpression defined as intensity of 2+ or 3+.
†As determined by FISH of tissue microarray sections. ''EGFR FISH'', determination of FISH-positivity (aneusomy with ≥40% of cells having ≥4 copies of *EGFR* or *EGFR* amplification); ''HER2 FISH,'' determination of *Her-2/neu* amplification.
‡As determined by immunohistochemistry of tissue microarray sections.
§Recurrent disease; all other cases were primaries.

amplification by chromogenic *in situ* hybridization (''15 gene copies/nucleus''). Furthermore, this metaplastic tumor lacked activating *EGFR* mutations in the exons examined (19 and 21). Reis-Filho et al. (28) reported seven metaplastic breast carcinomas that showed by chromogenic *in situ* hybridization apparently low-level amplification (''>5 signals/nuclei'') to high-level amplification (''large gene signal clusters''): four spindle cell and three carcinomas with squamous elements. No indication was given, however, as to which of the seven showed the high-level *EGFR* amplification essential for the high *EGFR* copy number associated with gefitinib response in NSCLC. This same group did a follow-up study enlarging the sample size to 47 metaplastic carcinomas and showed (by chromogenic *in situ* hybridization) some level of *EGFR* amplification in 11 tumors but no *EGFR* activating mutations (17). It should be noted that neither the report by Bhargava et al. (27) nor the two studies by Reis-Filho et al. (17, 28) labeled the chromosome 7 centromere in addition to the *EGFR* gene during chromogenic *in situ* hybridization analyses. This is vitally important, as it allows the calculation of the ratio of number of *EGFR* copies to number of chromosome 7 copies per sample, as was done in the FISH analyses presented here. Thus, this report is the first description in metaplastic breast carcinoma samples of an increase in *EGFR* copy number due to a balanced increase in the number of chromosome 7 copies. Furthermore, in the present study, only one of the metaplastic carcinomas with high *EGFR* copy number displayed *EGFR* gene amplification—the majority of the FISH-positive tumors (seven) showed high aneusomy in which at least 40% of the cells contained at least 4 copies of the *EGFR* gene (Fig. 2). These findings suggest that *EGFR* amplification is rare in metaplastic breast carcinomas, and that aneusomy is the most likely mechanism for high *EGFR* copy number.

As has been seen for HER2-overexpression in breast tumors (22), a direct correlation between EGFR immunohistochemistry and *EGFR* FISH-positivity in the metaplastic breast carcinomas in this study was not consistently found. As indicated in Table 4, the FISH-positive metaplastic samples ranged from 1+ to 3+ in immunohistochemical staining intensity, where the 1+ tumor would be considered negative for EGFR-overexpression. Thus, immunohistochemical measurements of EGFR protein expression did not prove to be predictive of FISH results determining *EGFR* copy number in metaplastic breast carcinomas.

Finally, as also indicated in Table 4, seven of the eight metaplastic breast carcinomas with high *EGFR* copy number contained a spindle cell component. Although more than one element in these seven tumors may have had high *EGFR* copy number, the spindle cell component was (except in one case) always FISH-positive. These data suggest that the spindle cell element was important for *EGFR* FISH-positivity in these tumors.

Unlike the relatively infrequent classical *EGFR* mutations in NSCLC, activating *KIT* mutations occur in as many as 90% of gastrointestinal stromal tumors, predominantly in exons 9 and 11 (see ref. 29 for review). *KIT* activating mutations have not been readily found in other tumor types (30–32). In a breast cancer study, 3% of 1,654 tumors examined were KIT-positive, and mutational analysis of 10 of the strongly positive tumors was negative for *KIT* activating mutations in exons 2, 8, 9, 11, 13, and 17 (33). Although together these studies suggest that *KIT* mutations are uncommon in tumors other than gastrointestinal stromal tumors, none specifically report *KIT* mutational analysis of metaplastic breast cancer, a member of the basal-like subclass of breast carcinomas of which >30% are KIT-positive (7). For the first time, we report that despite the presence of KIT expression in 24% of metaplastic breast tumors, *KIT* activating mutations were not present.

In summary, we have shown that metaplastic breast carcinomas exhibit molecular characteristics most consistent with the basal subtype of breast cancer. Although activating mutations in *EGFR* and *KIT* were not found, the

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presence of high *EGFR* copy number by FISH warrants further study to determine the role of EGFR tyrosine kinase inhibitors in treatment of metaplastic breast carcinoma patients.

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