EGFR Gene Copy Number Heterogeneity in Fine-Needle Aspiration Cytology From Breast Carcinomas Determined by Chromogenic In Situ Hybridization

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Most studies have shown epidermal growth factor receptor (EGFR) overexpression to be associated with poor prognostic factors in breast carcinomas. The relationship to EGFR gene copy number is unclear. The aim of our study was to investigate the heterogeneity of the EGFR gene copy number in breast carcinomas. The material consisted of air-dried smears from 29 breast carcinomas and 3 breast cancer cell lines (MCF-7, SKBR3, and T47D). Chromogenic in situ hybridization (CISH) was done using chromogenic detection. The mean signal numbers for EGFR gene and chromosome 7 as well as the EGFR gene/chromosome 7 centromere probe (CEP7) ratio were recorded. Immunohistochemical (IHC) staining was done on the corresponding paraffin sections.

The copy number of the EGFR gene in each tumor/cell line ranged from 1.2 to 5.6. The EGFR gene/CEP7 ratio showed a biological continuum ranging from 0.59 to 1.94 with a mean of 1.04. EGFR gene copy loss was found in 16.6% of cases whereas copy gain was demonstrated in 19.4%. There was no relationship between IHC protein expression of EGFR and EGFR gene copy number or EGFR gene/CEP7 ratio.

In conclusion, most breast carcinomas had a balanced EGFR gene/CEP7 copy number with a mean ratio of 1.04. Almost equal subpopulations revealed limited copy gain and copy loss. EGFR high dosage amplification, like in HER-2, was not demonstrated. Demonstration of EGFR gene copy loss might have a potential as a surrogate marker for EGFR gene mutation and/or deletion. Diagn. Cytopathol. 2005;33:228–232.

Key Words: EGFR; breast carcinoma; in situ hybridization; CISH; amplification

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This paper was presented at the 30th European Congress in Cytology in Athens in October 2004

Contract grant sponsor: Ullevålsfondet, Faculty Division Ullevål University Hospital, Oslo.

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Received 16 September 2004; Accepted 11 April 2005
DOI 10.1002/dc.20338
Published online in Wiley InterScience (www.interscience.wiley.com).

Several studies have investigated the significance of epidermal growth factor receptor (EGFR) in breast carcinomas. Increased expression has been reported from 20%[1,2] to 80%[3] in breast cancers.[4–18] Most studies have shown EGFR expression to be associated with poor prognostic factors.[2,4,6,7,9,11,12,14,15,18–20] Specific anti-EGFR agents as signal transduction inhibitors and monoclonal antibodies are currently being tested in clinical trials.[21,22] The relationship between EGFR expression in the breast carcinoma cells and the response to these agents is unclear.

The EGFR gene has been mapped to 7p13.[23] In breast cell lines, the EGF receptor has been found to play a major role in malignant transformation, with loss of EGF sensitivity and acquisition of an extra chromosome 7p harboring the EGFR gene.[24] Loss of heterozygosity at 7q has been found in 19–40% of breast cancers,[25–28] but not on the short arm where the EGFR gene is located. Numerical gain of chromosome 7 is a common finding occurring in about 60% of breast cancers,[29] but seems to have no impact on the EGFR expression.[30]

Mechanisms responsible for elevated EGFR could include both gene amplification and overexpression in the absence of gene amplification.[31–33] In breast cell lines, gene amplification appears to be a rare event[24] and differences in expression seems to be controlled to a great extent at the transcriptional level.[31,34] This would be in concordance with other studies finding that the EGFR expression is mainly regulated at the mRNA level and probably due to a direct effect of estrogen on the EGFR gene.[35,36] Amplifications of the EGFR gene have been demonstrated in several other types of tumors.[37–41] The aim of our study was to investigate the heterogeneity of the EGFR gene copy number in breast carcinoma cells and compare with the EGFR protein expression as well as the copy number of chromosome 7.

Table I. Details of EGFR and CEP7 Copy Numbers, Ratio, and Ploidy From FNAC Smears and Breast Cancer Cell Lines

Cell line or case no.	*Mean EGFR gene copy numbers*	*Mean CEP7 copy numbers*	*EGFR gene/CEP7 ratio*	*CEP7 ploidy*
T47D	3.9	6.6	0.59 (copy loss)	Aneusomic
1-5	1.97–2.4	2.2–3.6	0.64–0.7 (copy loss)	Disomic (1), anuesomic (4)
6–25	2.2–4.36	1.95–4.5	0.84–1.2 (balanced copy numbers)	Disomic (1), aneusomic (17)
SKBR3	5.69	5.41	1.05 (balanced copy numbers)	Aneusomic
MCF-7	3.3	3.1	1.06 (balanced copy numbers)	Aneusomic
26–32	2.6–5.02	1.52–3.26	1.3–1.94 (copy gain)	

Materials and Methods

The material consisted of air-dried smears from 29 breast carcinomas and the 3 breast cancer cell lines MCF-7, SKBR-3, and T47D. The smears had been kept at −20°C until processing and were fixed in methanol/acetic acid (3:1) prior to chromogenic in situ hybridization (CISH). CISH was done manually using the Spot-Light™ EGFR DNA probe with the CISH™ Polymer detection kit, as well as the Spot-Light™ chromosome 7 centromere probe (CEP7) and CISH™ Centromere detection kit (both from ZYMED laboratories Inc, San Francisco, USA). The procedures were done according to the manufacturer's recommendations. Signals were counted in at least 100 tumor cell nuclei. Only clearly identifiable, nonoverlapping nuclei were counted. The mean signal numbers for EGFR gene and chromosome 7 as well as the EGFR gene/CEP7 ratio were recorded. A ratio of 0.8–1.2 was considered to indicate an equal number of gene copies versus the chromosome centromere copies, according to findings in a previous study.[42] Lower and higher rates were considered as loss and gain of EGFR gene copy numbers, respectively. Smears from a fibroadenoma were used as benign control.

IHC staining was done on the paraffin sections using a monoclonal antibody against EGFR (EGFR pharmDx®, DakoCytomation, Denmark). The staining was performed on the DAKO Autostainer, using the dextran-polymer technique and with diaminobenzidine (DAB) as visualization. Both membrane and cytoplasmic staining were evaluated according to the manufacturer's recommendations, and both were recorded as positive. Normal skin, known positive cases as well as the manufacturer's positive control cells were used as controls.

Results

The details of the EGFR gene and CEP7 copy numbers are given in Table I. The mean copy number of the EGFR gene in each tumor/cell line ranged from 1.2 to 5.6. The mean CEP7 copy number ranged from 1.5 to 6.6. Of these, six cases showed disomy, whereas the rest were aneusomic. The EGFR gene/CEP7 ratio ranged from 0.59 to 1.94. Copy gain (Figs. C-1 and C-2) up to a doubling of the EGFR gene numbers was found in seven cases (19.4%). The majority of the tumors (64%, 23 cases) had equal copy numbers (Figs. C-3 and C-4) of the EGFR gene and CEP7, whereas six cases (16.6%) revealed limited loss (Figs. C-5 and C-6) of EGFR copy numbers. The most distinct EGFR gene copy loss was found in the T47D cell line. EGFR gene copy number showed no relationship to chromosome 7 ploidy. The total mean EGFR gene signal number was 3.0, the total mean CEP7 signal number was 3.1, and the mean EGFR gene/CEP7 ratio was 1.04. The fibroadenoma had an EGFR gene/CEP7 ratio of 1.06.

IHC EGFR status was obtained in 25 cases, of which 6 were positive (24%). There was no relationship between IHC EGFR and EGFR gene copy number and EGFR gene/CEP7 ratio.

Discussion

We found a distinct heterogeneity of the EGFR gene copy numbers in the carcinoma cells and cell lines. Basically it reflected the heterogeneity of the chromosome 7 copy number shown in the mean of EGFR copy numbers (3.0), the mean of CEP7 copy numbers (3.1), and the mean ratio between the two (1.04). In addition, there were almost equal numbers of cases revealing limited EGFR gene copy gain or copy loss. These findings were reflected in the EGFR gene/CEP7 ratio, which showed a biological continuum from 0.59 to 1.94.

Finding gain of EGFR gene copy number is in accordance with previous studies that suggest that malignant transformed breast cell lines acquire an extra 7p.[25] A limited copy gain, or low dosage amplification, was demonstrated in 19.4% of cases. This is somewhat higher than that observed by Kersting et al.,[43] but may be due to selection bias. CISH was done on cases with additional, archival (unstained) smears, and low-grade carcinomas are underrepresented. Kersting et al.[43] found a 2–4-fold amplification by RT-PCR, whereas the level of amplification by FISH was not described. They too found little correlation with IHC EGFR expression. Furthermore, they did not describe any EGFR gene copy loss.

Hirsch et al.[44] described EGFR gene amplification in 9% of non-small-cell lung carcinomas. The rest of their tumors showed balanced disomy (40%), balanced trisomy (38%), and balanced aneusomy (13%). Copy loss was not reported. Similarly, Marquez et al.[45] found amplification

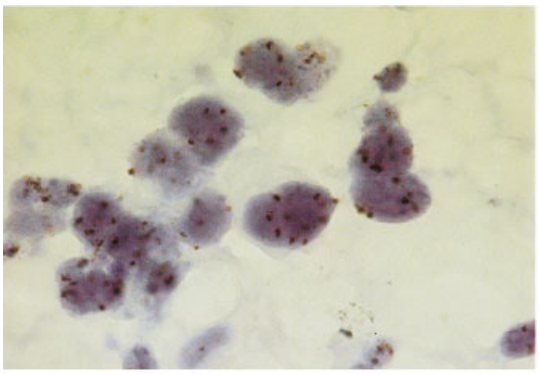

Fig. C-1

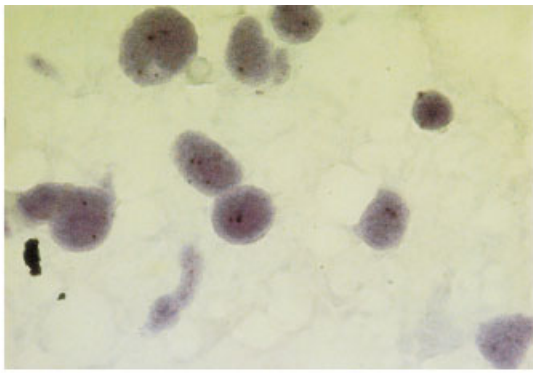

Fig. C-2

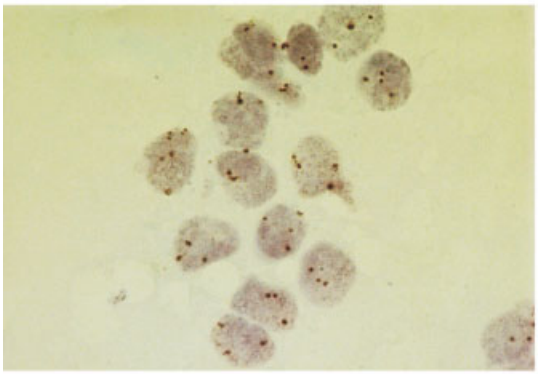

Fig. C-3

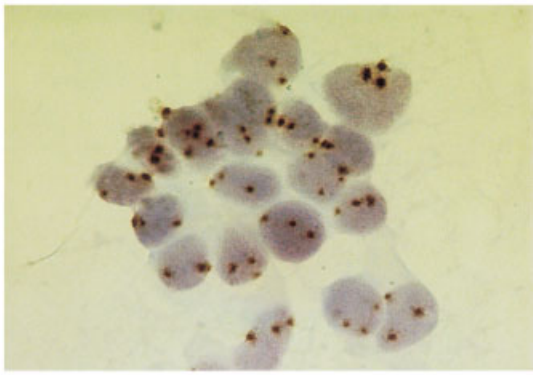

Fig. C-4

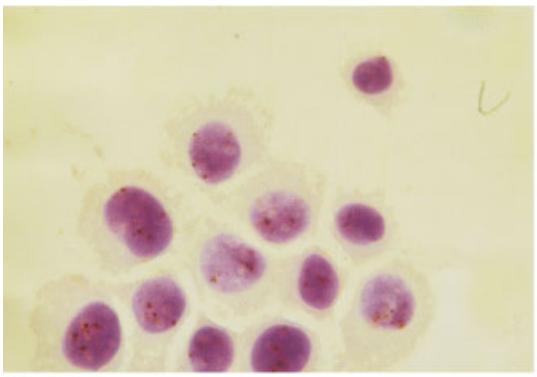

Fig. C-5

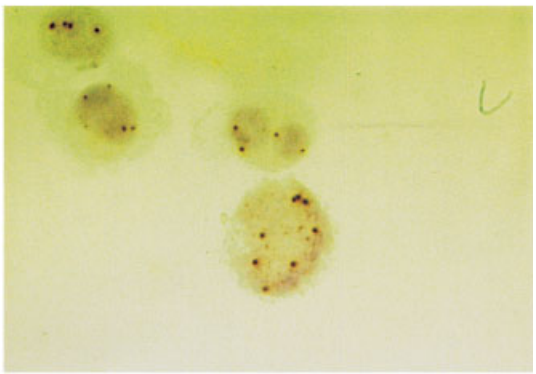

Fig. C-6

Figs. C-1–C-6. Fig. C-1. Breast carcinoma cells with EGFR gene copy gain (original magnification × 100). **Fig. C-2.** Chromosome 7 copy numbers, same as in Figure C-1 (original magnification × 100). **Fig. C-3.** EGFR gene copy numbers in breast carcinoma cells with EGFR gene/CEP7 ratio of approximately 1 (original magnification × 100). **Fig. C-4.** Chromosome 7 copy numbers, same as in Figure C-3 (original magnification × 100). **Fig. C-5.** EGFR gene copy loss in breast carcinoma cell line T47D (original magnification × 100). **Fig. C-6.** Chromosome 7 copy number in breast carcinoma cell line T47D, same as in Figure C-5 (original magnification × 100).

in 15% of gliomas, otherwise balanced disomy or aneusomy, but no copy loss. Ooi et al.[46] described EGFR gene amplification in colorectal cancer, but no copy loss.

Copy loss may involve mutations and/or deletions of the EGFR gene. The significance of gene copy loss in breast carcinomas is unknown. Two recent studies have shown that EGFR mutation in non-small-cell lung cancer correlated with clinical response to Gefitinib therapy.[47,48] The authors also found gene mutation in 8% and 14% (pooled results from two populations) of unselected cases or cases not exposed to Gefitinib, respectively. The EGFR gene changes may well be different in non-small-cell lung carcinomas and breast carcinomas, but our 16.6% cases with copy loss match the findings in the two studies rather well. Also, Pao et al.[49] found EGFR gene mutations in 7 of 10 Gefinitib-sensitive lung carcinomas, but none in Gefinitib-refractory tumors. They also found analogous mutations in 5 of 7 Erlotinib (Tarceva™)-sensitive tumors and in none of 10 Erlotinib-refractory carcinomas. Most of these were adenocarcinomas.

Butt et al.[50] found distinct growth inhibition of T47D cells by blocking of EGFR kinase activity. We found a distinct EGFR copy loss in T47D cells (Table I, Figs. C-5 and C-6), supporting the possibility that the mechanism of anti-EGFR sensitivity might be the same in breast carcinomas.

In contrast to HER-2,[51–57] EGFR gene expression is not reflected in the IHC protein expression. The mechanisms of EGFR activation and signaling are complex,[58] but seems to be unrelated to the gene copy numbers. Also unlike HER-2, high dosage gene amplification of the EGFR gene does not occur. Only a minor copy gain up to doubling of the gene copy numbers could be demonstrated. EGFR and HER-2 belong to the same family of tyrosine kinase receptors. They have 70% structural homology and frequently heterodimerize.[59] Yet their mechanisms of activation and action are distinctly different. Anti-EGFR drugs are promising anticancer agents, but highly predictive or surrogate markers are lacking. Neither EGFR gene amplification/copy gain nor IHC expression has the same predictive value as seen with HER-2, and other predictive markers need to be found.

Most of the research on EGFR and response to anti-EGFR therapy has concentrated on amplification and protein expression in the anticipation that the mechanism of response to specific anti-EGFR therapy would be similar to HER-2. The results from Lynch et al.[47] and Paez et al.[48] indicate that future studies should focus on mutation/deletion of the EGFR gene. Whether EGFR copy loss corresponds to gene mutations and/or deletions remains to be demonstrated. Is so, demonstration of EGFR gene copy loss might act as a surrogate marker for EGFR gene mutations and/or deletions.

In conclusion, most breast carcinomas had a balanced EGFR gene/CEP7 copy number with a mean ratio between the two of 1.04. Almost equal subpopulations revealed limited copy gain (19.4%) and copy loss (16.6%). EGFR high dosage amplification, like in HER-2, was not demonstrated in this study. Demonstration of EGFR gene copy loss might have a potential as a surrogate marker for EGFR gene mutation and/or deletion.

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