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Advances in Brief

Length and Loss of Heterozygosity of an Intron 1 Polymorphic Sequence of *egfr* Is Related to Cytogenetic Alterations and Epithelial Growth Factor Receptor Expression[1]

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Abstract

Overexpression of epithelial growth factor receptor (EGFR) is correlated with a poor prognosis and reduced steroid receptor expression. Recently, it was demonstrated that the length of a CA repeat in the intron 1 of EGFR correlated with the expression of EGFR *in vitro*. We investigated 112 cases of cancerous and noncancerous breast tumor samples for loss of heterozygosity (LOH) in intron 1 of the *egfr* gene and determined the intratumoral EGFR content and genetic alterations by comparative genomic hybridization. Heterozygous tumors with short CA repeats showed elevated EGFR expression in contrast to tumors with longer CA repeats. Tumors with LOH in intron 1 of *egfr* revealed higher EGFR expression when the longer allele was lost compared with loss of the shorter allele. Additionally, tumors with a loss of the long allele showed more chromosomal alterations, especially a higher frequency of amplifications. We conclude that the CA repeat status in intron 1 of the *egfr* gene also modulates the intratumoral EGFR content *in vivo*. Furthermore, LOH at the CA repeat is associated with genetically advanced tumors. Therefore, allele-specific gene expression due to LOH of the CA repeat could be assumed to be an important event in invasive breast cancer development.

Introduction

Invasive breast cancer is characterized by a broad spectrum of genetic alterations and heterogeneous morphological differentiation. The first evidence exists that different morphological subtypes might be associated with specific genetic alterations (1). A large variety of growth factors have been described in the pathogenesis and progression of invasive breast cancer. Proteins of the epithelial growth factor receptor superfamily are among the most investigated in this heterogeneous disease and are known to play a crucial role in the differentiation of the mammary gland (2). EGFR (erbB-1) stimulates cell growth and differentiation after binding of specific ligands, such as transforming growth factor-α (3). Thereby, EGFR[3] acts as membrane-bound receptor with intrinsic tyrosine kinase activity in the intracellular domain (4). Overexpression has been shown to transform NIH 3T3 cells in an EGF-dependent manner. Dimerization with other erbB receptors (erbB-2, -3, and -4) and the activation of the kinase domain are essential for phosphorylation of a variety of intracellular protein cascades. Overexpression of EGFR was inversely correlated with steroid receptor expression and positively with an unfavorable prognosis. In a study published by this group, a critical EGFR content of 56 fmol/mg was defined (5).

Overexpression of EGFR protein has been linked to amplifications of the *egfr* gene located to 7p13–12. In the majority of the tumors and especially in breast cancer, the main reason for overexpression has to be viewed at the gene transcription level (6). In a study published recently by this group, it was shown that the length of a highly polymorphic sequence in intron 1 of the *egfr* gene is directly correlated to the transcriptional activity of *egfr in vitro* and therefore might explain different expression levels of EGFR in humans (7).

Transferring these data to *in vivo*, this would lead to the hypothesis that humans with shorter alleles would show a higher level of EGFR expression in breast tumors and, furthermore, that LOH within this region would also lead to altered expression.

To investigate the relationship between the length of the polymorphic site within intron 1 to the expression level of EGFR, we performed microsatellite analysis on the polymorphic site close to intron 1, measured the content of EGFR by ELISA, and gained an overall indication of unbalanced chromosomal alterations using CGH.

Materials and Methods

Fresh tumorous material of 112 tumor samples from 103 patients was investigated. The tumorous material and reference lymphocytes were obtained from patients treated in the Department of Obstetrics and Gynecology of the University of Münster in the period of 1995–1997. All cases were classified according to standard protocols in the Gerhard-Domagk-Institute of Pathology, University of Münster. The cases were graded as ductal invasive ($n = 56$), poorly differentiated DCIS ($n = 3$), lobular invasive ($n = 18$), tumors of mixed differentiation ($n = 15$), tubular invasive ($n = 4$), tubulo-lobular invasive ($n = 3$), medullary invasive ($n = 1$), and mucinous invasive ($n = 1$). Seven cases of mastopathia, three samples of fibroadenomas, and one adenosis could be analyzed. In two cases, an associated DCIS located next to the invasive carcinoma and the corresponding lymph node metastasis of the same patient could be analyzed. In these cases, formalin-fixed and paraffin-embedded tissue was used. Staging was performed using the criteria of the tumor-node-metastasis system and of the Union International Contre Cancer. The age range of the patients was 18–85 years, with an average age of 60.2 years.

Steroid Receptor Immunohistochemistry. Immunohistochemical evaluation of the steroid receptor content was done according to standard protocols as described elsewhere (8).

EGFR Expression. The tumor samples were frozen in liquid nitrogen and homogenized in chilled 5 mM phosphate buffer containing 1 mM monothioglycerol and 10% glycerol (pH 7.5). An immunoenzymatic assay was used for the quantification of EGFR (EGF-Rezeptor ELISA; ImmunDiagnostik, Bensheim, Germany). The EGFR concentration was determined by comparison with recombinant standard material and expressed as fmol/mg membrane material.

Determination of the Number of CA Repeats in Intron 1. A 114- to 128-bp PCR fragment containing the polymorphic region was amplified with

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[3] The abbreviations used are: EGFR, epithelial growth factor receptor; CGH, comparative genomic hybridization; LOH, loss of heterozygosity; DCIS, ductal carcinoma *in situ*.

50 pmol of previously described primers (9). One of the primers was labeled with fluorescein at the 5′ end. The 50-μl PCR reaction mixture contained 200 ng of tumor DNA, 1.5 mM $MgCl_2$, 7.5% DMSO (Sigma), 100 μM each dNTP (Perkin-Elmer), 1× PCR amplification buffer, 1.5 units of Taq polymerase (Promega), and liquid white mineral oil (Sigma). After PCR, 1 μl of the products plus 0.3 μl of Genescan 500 TAMRA molecular weight standard (Applied Biosystems) were denatured in 12 μl of formamide, separated in an Applied Biosystems Prism Genetic Analyzer with POP4 polymer, and fragment lengths were determined. Genotyper software (PE) and Excel software (Microsoft) were used for allele scoring and assessment of LOH. The scans for constitutional heterozygous patients were analyzed by comparison of the peak area of the two alleles in the normal tissue (leukocytes) and in the tumor tissue by the following equation (9): LOH score $= T_1 \times N_2/T_2 \times N_1$, where *T* is tumor, *N* is normal, *1* is the area under the peak corresponding to the shorter allele, and *2* is the area under the peak corresponding to the longer allele. LOH assessment of the egfr microsatellite was not complicated or invalidated by extra bands microsatellite instability or stutter bands. The reproducibility of allele length and allele peak area evaluation was determined using 10 runs of PCR products from normal DNA from different persons' leukocytes. The SD for the allele length was 0.3 bp. For the ratio of the normal allele peak areas (N_2/N_1), the SD was 0.07. Therefore, the result was statistically significantly different from the ratio of the normal allele peak areas when the LOH score was <0.79 (loss of the longer allele) or >1.27 (loss of the shorter allele; Ref. 10).

CGH. The CGH analysis and the criteria for the evaluation of copy number changes have been described elsewhere (11). Tumor DNA (300 ng) was labeled by a standard nick-translation reaction with biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany). Reference DNA (300 ng) from a healthy female donor was labeled with digoxigenin-11-dUTP (Boehringer Mannheim).

The Cytovision 3.1 (Applied Imaging) software package was applied for digital image analysis and subsequent karyotyping.

Laser Microdissection. Tissue from two cases of DCIS with LOH in the associated invasive carcinoma was analyzed after laser-based microdissection (Palm, Bernried, Germany).

Statistical Analysis. For statistical analysis, the unpaired Student's *t* test and the χ^2 test were used.

Results

LOH of the Polymorphic Site of the EGFR Receptor in Intron 1. Eighty-two percent of the patients were informative for the locus investigated. No difference in patient age in relation to allele status or LOH was seen. In 57 patients, the smaller allele had a length of 16 CA repeats; in the remaining cases, the second most prevalent bp length was 20 CA repeats ($n = 17$). The most prevalent genotype comprised 16 and 20 CA repeats ($n = 22$).

Thirty-two patients showed a LOH; in 11 patients, the smaller allele was lost, in 21, the longer allele was lost ($P < 0.05$). One case of mastopathia and one case of an adenosis showed LOH. In two cases, the associated DCIS and the corresponding lymph node metastasis revealed the same LOH as the primary tumor (Fig. 1*B*). A correlation between the frequency of alleles or the occurrence of LOH and histology, grading, or tumor status could not be drawn.

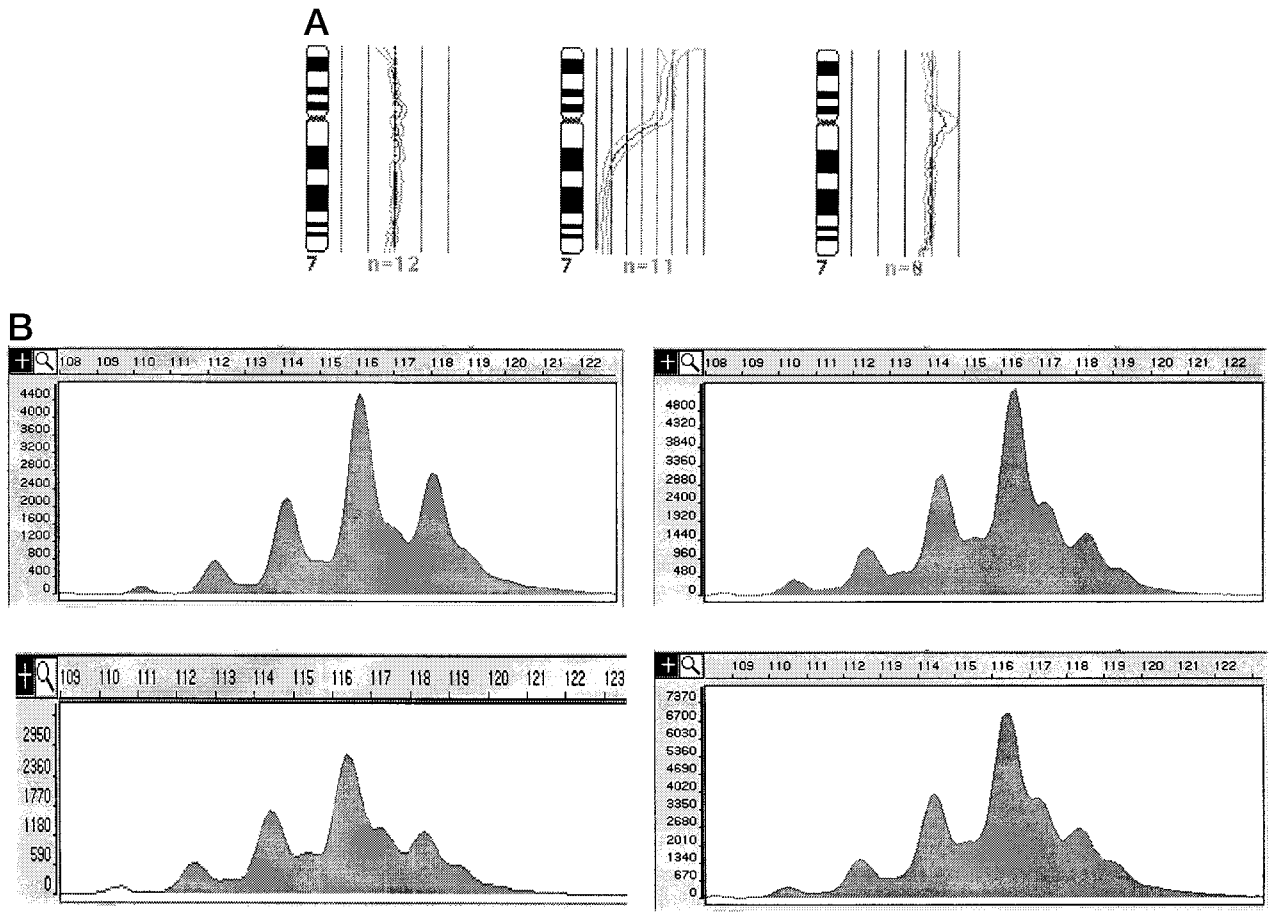

Fig. 1. *B*, examples of average ratio profiles of chromosome 7 alterations revealed by CGH. The *vertical broad central line* indicates the fluorescence ratio of balanced DNA sequence copy number state (1.0) between tumor and reference DNA. The *vertical lines* to the *left* represent the 0.75 and 0.5 thresholds for losses, the *vertical lines* to the *right* represent the 1.25, 1.5, and 1.75 thresholds for gains. The ratio profiles show the mean fluorescence ratio (*central*) and the 95% confidence limits (*left* and *right* of central line). Chromosome numbers are indicated. *B*, example of a LOH in intron 1 of the *egfr* gene. *Upper left*, heterozygosity in blood lymphocytes; *upper right*, LOH in invasive breast cancer of the same patient; *lower left*, LOH in associated DCIS; *lower right*, LOH in corresponding lymph node metastasis.

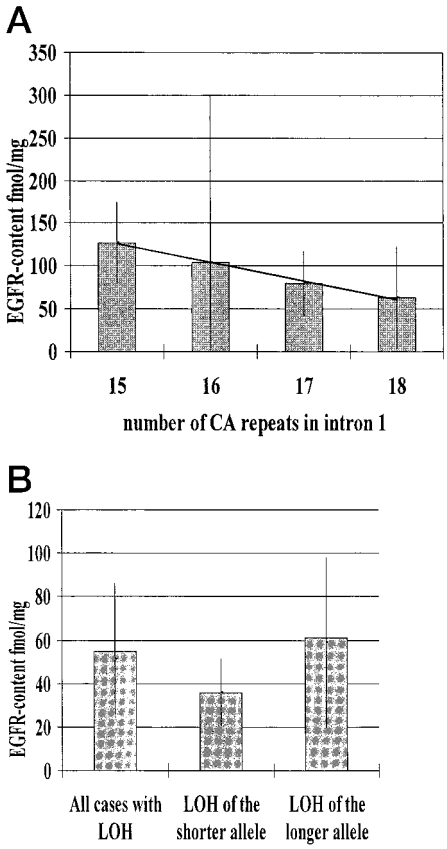

Fig. 2. *A*, graphical illustration of the influence of the smaller CA repeat allele on EGFR expression. The number of CA repeats in intron 1 is indicated on the *X axis*, the expression of EGFR on the *Y axis* (fmol/mg). Mean values with the corresponding standard deviations (*bars*) are indicated. *B*, illustration of the influence of LOH on EGFR expression with a defined example of a 16/20 CA repeat allele composition. *Columns* represent the median; *points* and *vertical lines* represent mean values and the corresponding standard deviations.

CGH Analysis and LOH. Forty-one cases of invasive breast cancer could be analyzed by means of CGH: 20 of them presented with LOH, 8 were homozygous, and the remaining 13 were heterozygous. Isolated losses of 7p could not be detected, irrespective of homozygous or heterozygous allele status or detected LOH. In nine cases, gains of the whole chromosome 7 or parts of 7p could be detected; two of these cases with a 7p-gain LOH could be detected. In regard to the average number of alterations and the rate of tumors with amplification, no significant difference between homozygous and heterozygous tumors could be seen. Tumors with a loss of the long allele showed, on average, more alterations per case compared with tumors with loss of the short allele (12.7 ± 6.7 *versus* 6.5 ± 3.9; $P < 0.05$) The rate of amplifications was also increased in tumors with loss of the longer allele (69 *versus* 14%; $P < 0.05$). However, a distinct genetic pattern of chromosomal alteration in tumors with LOH could not be detected. Representative profiles of chromosome 7p alterations are given in Fig. 1*A*.

EGFR Expression, Allele Length, and Hemizygosity. In all cases, heterozygous samples showed a lower level of intratumoral EGFR protein [76.8 ± 47.7 fmol/mg (mean $\pm$ SD); median, 68.4 fmol/mg; $n = 18$] in contrast to homozygous cases (224.47 ± 246.7 fmol/mg; median, 88.7 fmol/mg; $n = 56$; $P < 0.01$).

Regarding EGFR expression in correlation to the length of the smaller allele in all tumors, a constant decline in the intratumoral EGFR content could be demonstrated with an increasing length of the smaller allele (Fig. 2*A*). The highest levels were measured in tumors with a length of 15 CA repeats (127.5 ± 47.4 fmol/mg; $n = 6$), and the lowest levels were measured in tumors with 18 CA repeats (52.0 ± 59.1 fmol/mg; $n = 17$) as the smaller allele ($P < 0.05$).

In general, LOH was not correlated with higher EGFR expression. The samples with a loss of the longer allele showed higher EGFR content in contrast to the tumors with the longer allele remaining. An example for the relationship of LOH and EGFR expression for the case heterozygotes with 16 and 22 CA repeats is given in Fig. 2*B*. When the 16-CA repeat allele was lost, there was a tentative lower intratumoral protein concentration than when the longer allele was lost (36.1 ± 15.6 fmol/mg; median, 36.3 fmol/mg *versus* 59.18 ± 39.72 fmol/mg; median, 61.8 fmol/mg).

Cases with a prognostically unfavorable intratumoral EGFR content of >56 fmol/mg ($n = 41$) showed almost exclusively a short allele with 16–18 CA repeats. Twelve of these cases revealed LOH, 9 of them with a loss of the longer allele.

EGFR Expression and Steroid Receptor Immunohistochemistry. Twenty-four tumors showed no expression of the estrogen receptor, 6 of them with LOH. The EGFR content was elevated (mean $\pm$ SD, 119.4 ± 137.7 fmol/mg) in these tumors. Twenty-seven tumors were negative for progesterone receptor, 8 of them with LOH in intron 1. Here also an elevated EGFR concentration could be demonstrated (mean $\pm$ SD, 143.3 ± 155.5 fmol/mg).

Discussion

In human breast cancer, overexpression of EGFR is associated with short disease-free and overall survival. As shown in several studies, low estrogen receptor expression was inversely related to EGFR expression (12). The background of this finding and the genetic basis of EGFR overexpression remain unclear. Thus far, amplifications of the *egfr* gene (7p13) has been revealed only in a small subset of breast cancers (13). In *egfr*, the regulatory sequences of the gene have been demonstrated within the 5′-flanking region and intron 1. Two sequences with enhancer activity were located upstream of the promoter and downstream in intron 1 (14). Close to the downstream enhancer, a polymorphic simple sequence repeat of 14–22 CA repeats was found. In *egfr*, the number of CA repeats in intron 1 correlates with the *in vitro* transcription of the gene (7). In this study, we could show that these results can be transferred to a clinical background. The most prevalent allele composition in the population investigated is a 16/20 CA repeat combination. Interestingly, it is noteworthy that >90% of all women in this population presented with at least one short allele.[4] Loss of LOH, irrespective of which allele is lost, was not significantly correlated with altered EGFR expression. Nevertheless, LOH does not lead to the total suppression of the EGFR expression. In contrast, cases with a loss of the 16 CA repeat allele showed a lower intratumor protein concentration than those with a loss of the longer 22 CA repeat. This could be further substantiated in the rest of the tumors investigated. Tumors with homozygous allele status showed higher gene expression than heterozygous cases. This probably can be explained by the fact that in our series, the majority of the homozygous patients revealed a 16/16 CA repeat combination, and therefore this short allele was transcribed with higher activity. As well as in the defined subgroup mentioned above, all tumors with LOH of the longer allele revealed higher intratumoral EGFR content in contrast to the tumors with loss of the short allele. The influence of the polymorphic sequence can also be demonstrated in heterozygous tumors without LOH. In these cases, the effect is not as dramatic as in tumors with LOH or in homozygous samples, but the tendency is obvious. Regarding only the smaller allele as reference independent of LOH, EGFR expression constantly decreased with increasing length of the CA repeat in intron 1. The high standard deviations might explain that other mechanism in these heterozygous

[4] Unpublished data.

tumors also influence the expression of EGFR, as shown in previous *in vitro* studies [(7) and Fig. 2*A*].

Nevertheless, we interpret these results as first evidence that the *in vivo* expression of EGFR in a subset of breast cancer is correlated with the length of the CA repeat in intron 1 of the *egfr* gene. This finding is not only interesting in relation to tumor progression, it also could give a first clue to explain the function of microsatellites in correlation to the individually different gene expression. Therefore, the length of the CA repeat in intron 1 of the *egfr* gene might be one factor in the predisposition to human breast cancer. A significant predominance of LOH in intron 1 of the *egfr* gene in a certain morphological subgroup, such as ductal invasive or lobular invasive carcinoma, could not be detected. Additionally, LOH occurred independently of tumor grading and staging. This is different from *BRCA1*, where mutations were associated with grade 3 tumors, indicating a low differentiation. Nevertheless, these findings must be established in a larger cohort of tumor samples. Mutations in the *BRCA1* and *BRCA2* genes are reflected on a cytogenetic level. Breast cancer tissue with mutations in either of the genes showed an elevated average number of alterations as well as an increased rate of amplifications (15). The number of cases investigated in this series might be too small to draw definite conclusions. Nevertheless, the first results show similar results with an increase of the average number of alterations and the rate of tumors with gene amplifications. Tumors with loss of the longer allele showed almost five times more gene amplifications and an increase of the average number of genetic alterations.

Furthermore, it is of interest that a distinct polymorphism in intron 1 or LOH of one allele seems not to be associated with a distinct cytogenetic pathway, as described by our group earlier (1, 16). They also are not correlated with distinct genetic alterations, which furthermore stresses the hypothesis that allele-specific gene expression levels due to short polymorphic sequences or LOH are early factors in the carcinogenesis of human breast cancer. The hypothesis that LOH might be an early event in the carcinogenesis of breast cancer is sustained by the fact that LOH was detected in one case of an adenosis and one case of a mastopathia. Additionally, it could be shown in two cases with a DCIS–invasive carcinoma–metastasis sequence, that the LOH occurs in early premalignant tumors of the breast.

There is strong evidence that the increased expression of EGFR is not associated with gross genetic alterations involving chromosome 7p. On a cytogenetic level, amplifications involving the *egfr* gene locus are a rare event. In several studies, losses or isolated gains of chromosome 7p were found in $<5\%$ of all tumors investigated (1, 17, 18). Two of our cases revealed high-level gains of the whole short arm of chromosome 7. It is noteworthy that one of these tumors showed only low intratumoral EGFR content (19 fmol/mg); unfortunately, in the other tumor sample, protein extraction was not possible. Similar to other epithelial neoplasms, altered EGFR expression, therefore, is not reflected on the cytogenetic level (19), and microdeletions in the *egfr* gene play a much more important role in gene expression.

It has been speculated that reduced EGFR expression at the time of exposure to carcinogens might account for a higher resistance toward development of breast cancer in rats (20). The hypothetical combination of inherited CA repeats with different lengths and their direct influence on EGFR expression and further losses of CA repeats, possibly due to chemical carcinogenesis, might therefore bridge the gap between different genetic and epigenetic factors in the carcinogenesis of invasive breast cancer.

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