In Situ Hybridization of Chromosome 6 on Fine-Needle Aspirates from Breast Carcinomas: Comparison of Numerical Abnormalities and ER/PgR Status and Staining Pattern

Torill Sauer, M.D., F.I.A.C.,^{1*} Kahsai Beraki, B.Sc.,¹ Peter Wilhelm Jebsen, M.D.,¹ Eli Ormerod, B.Sc.,² and Oddvar Naess, M.D.¹

The estrogen receptor (ER) gene is located on chromosome 6. The aim of our study was to investigate whether numerical chromosomal aberrations were reflected in estrogen/progesterone receptor (PgR) status and staining pattern. Fine-needle aspirates from 51 breast carcinomas were investigated immunocytochemically for ER/PgR and by in situ hybridization technique using digoxigenin-labeled α -satellite probe for chromosome 6. Cases with $\geq 70\%$ two-signal nuclei were regarded as disome; the remaining tumors showed aneusomy with a variable number of signals. Aneusomy was found in 32 tumors (63%), whereas 19 (37%) had a normal number of chromosome 6. Chromosomal gain occurred in all aneusome cases except one. ER- and/or PgR-positive tumors had an equal distribution of disomy and aneusmy. Variable ER staining pattern or ER and/or PgR negativity was associated with numerical aberrations in chromosome 6 in 76% of the tumors. Cancers with uniform ER staining pattern all had normal chromosome number. Diagn. Cytopathol. 16:420–424, 1997. © 1997 Wiley-Liss, Inc.

Key Words: estrogen receptor; in situ hybridization; chromosome six; chromosomal numerical aberration

Breast cancer is the most common malignancy among women in the industrialized world. Several prognostic factors have been recognized (e.g., grade, lymph node status, estrogen-receptor (ER) and progesterone-receptor (PgR) status, ploidy, p53 suppressor gene, and several oncogenes). The ER/PgR status also has therapeutic implications in these patients. About 40% of ER-positive carcinomas will respond to antiestrogen therapy.¹ Defects in ER mechanisms may explain this lack of response.² Determina-

tion of ER-dependent markers, such as PgR or pS2, gives additional information, and approximately 80% of ER-positive/PgR-positive tumors will respond to hormonal therapy.³

Several defects in the receptor molecule have been identified that render the immunopositive ER nonfunctional. Defects in both the DNA binding region and the estradiol binding region have been found.^{4–13} As significant mutations in the ER gene seem to be rare,^{14,15} the defects in ER function may be due to changes at the transcriptional or post-transcriptional level. The ER gene is located on chromosome 6q25.1.^{16,17} By applying in situ hybridization (ISH) to interphase nuclei, numerical aberrations in specific chromosomes may be visualized. The technique is well suited for material obtained by fine-needle aspiration (FNA),¹⁸ and both fluorescent (FISH)¹⁹ and nonfluorescent methods may be used.

Determination of ER/PgR status on FNAs, frozen sections, or paraffin-embedded material allows evaluation of heterogeneity in the staining of breast cancer cells. Nuclear staining mosaicism may reflect defect receptor modulation due to various structural modifications of receptor domains.²⁰

The aim of our study was to investigate whether abnormal numbers of chromosome 6 were reflected in ER (and PgR) status and staining pattern.

Materials and Methods

The material consists of FNAs from 51 patients with palpable carcinomas of the breast. The aspirations were performed by two cytopathologists (PWJ and TS). FNA is a routine procedure at our institution and part of the triple

¹Department of Pathology, Ullevaal University Hospital, Oslo, Norway

²Department of Medical Genetics, Ullevaal University Hospital, Oslo, Norway

*Correspondence to: Torill Sauer, Department of Pathology, Ullevaal University Hospital, N-0407 Oslo, Norway.

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Table I. Estrogen/Progesterone Receptor Staining

Staining intensity: weak—moderate—heavy
Staining pattern: uniform (all nuclei show the same staining intensity)—variable (staining intensity varies from one nucleus to the other)
Percentage of positive nuclei: $\leq 10\%$ = negative $> 10\%$ = positive (% given in report)

Table II. ISH Staining Procedure

1. Fix aspirates in methanol/acetic acid (3:1) for 40 min at -20°C
2. Dehydrate at room temperature (RT) in 70, 80, and 96% alcohol; allow to dry
3. Denature $2 \times \text{SSC}$, 70% formamide at pH 7.0 and 70°C for 2 min
4. Dehydrate at -20°C ; 2 min each in 70, 80, and 96% alcohol; allow to dry
5. Denature 5 min in water bath at 70°C
6. Add probe solution; incubate at 37°C for 16 hr (overnight)
7. Wash posthybridization ($0.20 \times \text{SSC}$; pH 7.0) at 72°C for 5 min
8. Wash in PBS (with 0.25% Triton-X 100) for 2 min at RT
9. Incubate with mouse-anti-digoxigenin for 3 hr at RT
10. Incubate with biotinylated anti-mouse (ABC kit) for 30 min at RT
11. Wash with TBS, 5 min at RT
12. Incubate with ABC complex for 1 hr at RT
13. Use DAB solution 10 min
14. Wash in running tap water, counterstain with hematoxylin and mount

Table III. Example of Recording of Signal Number in Three Different Tumors as Counted by ISH

<i>Number of signals pr. nuclei</i>	1	2	3	4	5	6	7	8	≥ 9
A	4.5%	80%	2.5%	12%	1%				
B	1%	27%	9%	31%	29%	2%	1%		
C		12%	1%	20%	16%	15%	11%	10%	15%

approach when investigating palpable breast lesions. In addition to diagnostic smears, we routinely perform ER and PgR determination on aspirates. Three smears are immediately placed in a box containing dry ice. In the laboratory, the smears are stored at -70°C until staining is performed. ER and PgR status are determined using the Abbott ER-ICA kit. Staining intensity, staining pattern, and the percentage of positively stained nuclei are recorded according to a semi-quantitative score, as shown in Table I.

In cases in which additional air-dried smears were available, these were put aside for ISH and kept at -20°C until processing. ISH was performed using a digoxigenin-labeled α -satellite probe for chromosome 6 (D6Z1 from Oncor). The staining procedure (according to Oncor with some modifications) is shown in Table II. An avidin-biotin complex (ABC) technique with diaminobenzidine (DAB) as substrate was used for visualization of the signals. The nonfluorescent staining used has the advantage of being permanent with no fading of signals. The specimens may be looked at in the light microscope at any time. The hematoxylin-eosin (H&E) counterstain allows us to make sure that only malignant nuclei are evaluated. The number of signals in 100 nuclei

Table IV. Correlation between ER/PgR Status and Staining Pattern and Numerical Aberrations in Chromosome 6

<i>Estrogen/progesterone receptor</i>	<i>Chromosome 6</i>		
	<i>Disomy</i>	<i>Aneusomy</i>	<i>Total</i>
ER-positive; <i>uniform</i> staining pattern	9		9
ER-positive; <i>variable</i> staining pattern	6	17	23
ER-negative	4	15	19
PgR-positive	13	11	24
uniform	2	1	3
variable	11	10	21
PgR-negative	6	20	26
ER-pos/PgR-pos	11	11	22
ER-pos/PgR-neg	4	5	9
ER-neg/PgR-pos	1	1	2
ER-neg/PgR-neg	3	14	17

Table V. Correlation between Abnormal Number of Chromosome 6 and Percentage of Positive Nuclei in ER-Positive Tumors

<i>Estrogen receptor Percentage of positive nuclei</i>	<i>Chromosome 6</i>	
	<i>Disomy</i>	<i>Aneusomy</i>
$\geq 75\%$	11	11
$< 75\%$	4	6

Total = 32.

were counted in each tumor, and the percentage of cells with varying signals was recorded (Table III). Only nuclei with clearly discernible signals were counted. Nuclei without signals were rarely seen in technically adequate areas of the smears. Cases with $\geq 70\%$ two-signal nuclei were regarded as disome and include those with a second four-signal peak (record type A in Table III). The remaining tumors showed aneusomy (aneuploidy), with a variable number of signals in the nuclei (record type B and C in Table III). Most aneusome carcinomas were of type B (28) with smaller peaks at two, four, and five chromosomes per nuclei, whereas a few (four) showed a more profound aneusmy with a wide distribution of signal numbers per nucleus. Three fibroadenomas and one benign intraductal papilloma were used as controls.

Results

Thirty-two (63%) carcinomas were aneusome, and 19 (37%) had a normal number of chromosome 6. All four benign tumors showed disomy with $> 90\%$ two-signal nuclei. In aneusome lesions, chromosomal gain was the dominant event, occurring in all but one case. The latter showed monosomy with $> 90\%$ one-signal nuclei (Fig. C-1). The distribution of signals varied. In 28 cases, nuclei with two, three, four, and five signals comprised the major part of the cell population (record type B in Table III), whereas four had a continuous distribution of signal number from one to more than nine (record type C in Table III). The results of ER/PgR status, staining pattern, and chromosome findings are shown in Table IV. Thirty-two (62.7%) tumors were ER-positive. The percentage of ER-positive nuclei did not correlate with numerical abnormalities in chromosome 6 (Table V). ER-

Fig. C-1A

Fig. C-1B, C

Fig. C-2A

Fig. C-2B, C

Fig. C-3A

Fig. C-3B, C

Figs. C1–C3. **Fig. C-1.** Breast carcinoma cells (×500). **A:** ISH showing most nuclei with one signal only (monosomy) for chromosome 6. **B** and **C:** Same tumor; ER and PgR both positive with variable staining pattern. **Fig. C-2.** Breast cancer cells (×500). **A:** ISH showing most nuclei to harbor two signals for chromosome 6 (disomy). **B:** Same tumor; ER positive and with a uniform staining pattern. **C:** Same tumor, but from metastatic axillary lymph node; PgR negative. **Fig. C-3.** Carcinoma cells (×500). **A:** ISH showing nuclei with a varying signal number (aneusomy). **B** and **C:** Same tumor; ER and PgR both negative.

positive carcinomas with a uniform staining pattern were all disome ($9 = 18\%$) (Fig. C-2). Seventeen of 23 tumors with a variable staining intensity and 15 of 19 ER-negative cases showed aneusomy (Fig. C-3). PgR positivity was found in 24 tumors. PgR staining pattern was not related to abnormal numbers of chromosome 6. PgR-negative and ER-negative/PgR-negative cancers had numerical aberrations in 77% and 82% of cases, respectively. PgR positivity and all other combinations of ER/PgR findings were found equally in tumors with normal and abnormal numbers of chromosome 6. Among the 12 cases with a uniform staining pattern, 10 ($ER = 7$; $PgR = 3$) had $>90\%$ stained nuclei, and two had about 50% positive cells. One PgR staining was weak, and the others showed a moderate or heavy staining. The cancers with a variable staining intensity (weak to heavy) showed distinct divergence in the proportion of positive nuclei, ranging from 25% to $>90\%$, but with the majority showing $\geq 75\%$.

Discussion

Most breast cancers have abnormal DNA content. This feature is reflected in our material, in which almost two-thirds of the carcinomas were aneusome for chromosome 6. As we used additionally available smears only, these specimens will not represent the full range of breast cancer types and low-grade carcinomas are probably underrepresented. The percentage of ER-positive cases (62.7%) indicates the same; the ER positivity in our total breast aspirates is 15% higher.²¹

Chromosomal gain was the most common event, occurring in 61% of the cases. Of the ER-positive tumors, 48% had an increased number of chromosome 6. Nembrot et al.²² found ER gene amplification in 43% of ER-positive carcinomas. However, they found no gene amplification in ER-negative cases, in contrast to 78% aneusomy in ER-negative tumors in our material. Gene amplification may result from repeated gene sequences on a chromosome or from chromosomal gain. The probe used here is directed toward the centromere region and mutations or deletions not affecting the centromere cannot be detected. Even if the chromosome number is increased, the ER gene sequence may be deleted and thus not appear as amplified.²³ Magdelat et al.²⁴ found that major variations in ER and PgR expression were independent of the number of copies of the corresponding gene. Our results are in line with that as far as ER- and/or PgR-positive tumors are concerned. These cases were equally distributed between normal and increased numbers of chromosome 6. However, in ER- and/or PgR-negative cancers, more than 75% had chromosomal gain.

In the management of breast cancer patients, not only positivity/negativity but also the amount of ER/PgR is of relevance, as higher hormone receptor values in tumors render them more likely to respond to hormonal therapy. So far, the staining pattern has not been paid much attention,

and in biochemical assay such properties are, of course, impossible to evaluate. The percentage of positive nuclei in ER-positive cancers was found to be equal in disome and aneusome tumors.²² The single monosome case had about 90% positive nuclei and a variable staining pattern of both ER and PgR (Fig. C-1). Although the amount of receptor-positive cells may to some extent predict response to therapy, this parameter gives no information about numerical aberrations in chromosome 6.

An interesting finding was that all nine ER-positive carcinomas with a uniform ER staining pattern had a normal number of chromosome 6. Eight of the tumors were also disome for the chromosomes 7, 12, and 17, whereas the last showed monosomy for chromosome 17.²⁵ This feature would be in good agreement with the concept that uniform staining indicates normal ER regulation. Two of the nine were PgR-negative (Fig. C-2), six had a variable, and only one a uniform PgR staining pattern. Even with normal ER regulation, PgR expression may be low and eventually not demonstrable when not directly stimulated by estrogen. Therefore, PgR negativity may not always be an indicator of an abnormal ER. Because of these diversities, however, we would hesitate to conclude that these specific tumor cells had a totally normal regulation of ER, albeit functionally active. In conclusion, a variable ER staining pattern or ER and/or PgR negativity is a strong indication of a tumor that has an increased number of chromosome 6. Furthermore, a uniform ER staining pattern seems to be characteristic of a carcinoma that is disome for chromosome 6. Finally, neither ER and/or PgR positivity nor percentage of ER-positive nuclei correlates with numerical aberrations of chromosome 6.

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