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ORIGINAL ARTICLE

Yanming Zhang · Reiner Siebert · Peter Matthiesen
Svetlana Harder · Michael Theile · Siegfried Scherneck
Brigitte Schlegelberger

Feasibility of simultaneous fluorescence immunophenotyping and fluorescence in situ hybridization study for the detection of estrogen receptor expression and deletions of the estrogen receptor gene in breast carcinoma cell lines

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Abstract For the first time, combined immunophenotyping and fluorescence in situ hybridization (FISH) technique according to the "fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms" (FICTION) technique have been successfully applied in solid tumors. Thus, we were able to visualize the antigen expression of cells with chromosomal deletions of a tumor suppressor region directly. In six breast carcinoma cell lines, we investigated the correlation between estrogen receptor (ER) expression status and deletions of the estrogen receptor gene (ESR). To screen for deletions of the ESR gene, dual-color FISH was performed with a YAC (yeast artificial chromosome) probe containing the ESR gene and, as internal control, with a centromeric probe of chromosome 6. Deletions of the ESR gene were detected in four of six cell lines. For direct comparison of ER expression with the copy number of the ESR gene at the single cell level, immunophenotyping with mouse anti-human ER antibody was combined with FISH with the YAC probe containing the ESR gene according to the FICTION technique. There was no correlation between lack of or reduced ER expression and deletions of the ESR gene. One cell line with deletions of the ESR gene did express ER on the protein level, while another cell line without a deletion did not. Cells with deletions of the ESR gene were either ER expression positive or negative. The staining intensity of ER expression was not associated with the copy number of the ESR gene. Thus, this FICTION study unequivocally shows that deletions of the ESR gene are not the major cause of absent or reduced ER expression in breast carcinoma cell lines.

Key words Immunophenotyping · FISH · Breast carcinoma · Deletions · ESR gene

Y. Zhang · R. Siebert · P. Matthiesen · S. Harder
B. Schlegelberger (✉)
Department of Human Genetics, University of Kiel,
Schwanenweg 24, D-24105 Kiel, Germany
e-mail: schlegelberger@medgen.uni-kiel.de
Tel.: +49-431-5971781, Fax: +49-431-5971880

M. Theile · S. Scherneck
Department of Tumor Genetics,
Max-Delbrück-Center for Molecular Medicine, Berlin, Germany

Introduction

Deletions of the long arm of chromosome 6 (6q) occur frequently in breast cancer, ovarian carcinoma and melanoma as well as in hematological neoplasms [2, 6, 22, 27]. Loss of heterozygosity (LOH) studies have shown 6q to be the second major site of LOH in breast cancer [5]. A commonly deleted region of 6q23–25.2 including the ESR (estrogen receptor) gene, which is localized at 6q25.1, was identified in microdissected breast cancer specimens [7]. Suppression of tumorigenesis and metastases of breast carcinoma cells after introduction of a normal chromosome 6 by microcell-mediated transfer further confirmed that one or more tumor suppressor genes responsible for the progression of breast carcinoma are localized on 6q [17, 26].

Estrogen receptor (ER) expression of breast carcinomas has been associated with response to treatment and with disease-free survival [8]. It is well known that the predominant factor determining ER expression is the activity of the array of promoters that control its expression rather than mutations or gene deletions. To demonstrate the feasibility of simultaneous fluorescence immunophenotyping and fluorescence in situ hybridization (FISH) according to the fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms (FICTION) technique [30] for the study of solid tumors, we investigated the correlation between ER expression status and deletions of the ESR gene in six breast carcinoma cell lines by directly combining immunophenotyping with a monoclonal mouse anti-human ER antibody and FISH with a YAC DNA

probe containing the ESR gene. No correlation between absent or reduced ER expression and deletions of the ESR gene was observed.

Materials and methods

Cell lines

Six breast carcinoma cell lines (BRC230, CAL51, MCF-7, MDA-MB-231, R103 and T-47D) were studied. MCF-7 and T-47D were known to be ER expression positive, while CAL51, BRC230 and MDA-MB-231 were negative [3, 9, 33].

Probes

The YAC 19I11F probe containing the ESR gene was obtained from ICRF (Cambridge, UK) [16]. The Alu-PCR product of the YAC probe was biotinylated using a commercial BioPrime labeling kit (Gibco/BRL, Rockville, Md.) according to the manufacturer's instructions. A digoxigenin-labeled D6Z1 probe specific for the centromeric region of chromosome 6 (Oncor, Gaithersburg, Md.) served as an internal control to determine the copy number of chromosome 6. For control studies, chromosome preparations from PHA-stimulated peripheral blood of five healthy donors were used.

Dual-color FISH

To screen for deletions of the ESR gene, dual-color FISH was performed as previously described [29]. The hybridization mixture contained 50% formamide, 10% dextran sulfate, 2×SSC, 10 µg/µl human Cot-1 DNA, 40 ng/µl YAC probe and 1 ng/µl D6Z1 probe. The biotinylated YAC 19I11F probe and the digoxigenin-labeled D6Z1 were visualized by Cy3 and FITC, respectively.

Combined immunophenotyping and FISH study

Combined immunophenotyping and FISH according to the FICTION technique was performed as previously described [30]. A monoclonal mouse anti-human ER antibody (Dakopatts, Hamburg, Germany) was used and was visualized by Cy3. After immunophenotyping the slides were fixed in 3:1 methanol:acetic acid fixative and in 1% paraformaldehyde, followed by dehydrating in a series of 70%, 85% and 100% ethanol. FISH with a biotinylated YAC 19I11F probe was performed as described above. The YAC probe was visualized by FITC.

For semi-quantitative determination of ER expression, T-47D, which is known to be ER expression positive, was used as positive control [3, 33]. Positive cells showed strong nuclear, but not cytoplasmic, red fluorescence. The staining intensity was graded into three levels: -, + and ++ [10, 14, 15]. If more than 20% of cells showed at least + level staining, the cell line concerned was considered to be ER expression positive [14, 24].

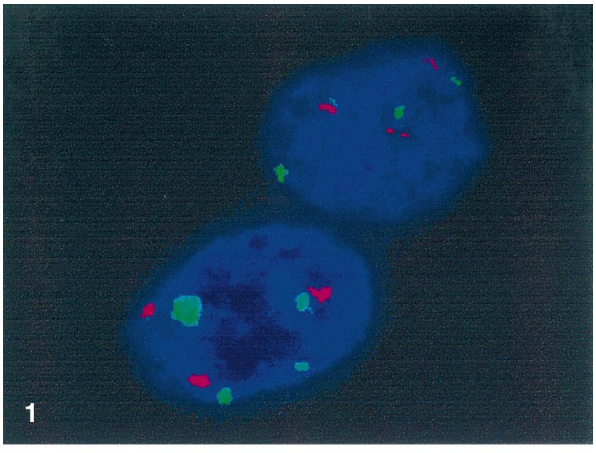
Evaluation and documentation

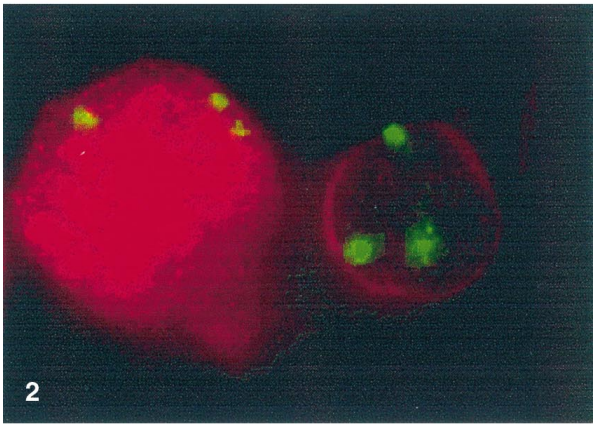
FISH and FICTION results were analyzed under a Zeiss fluorescence microscope with appropriate filter sets. For documentation, the ISIS imaging software (Metasystem, Althussheim, Germany) was used.

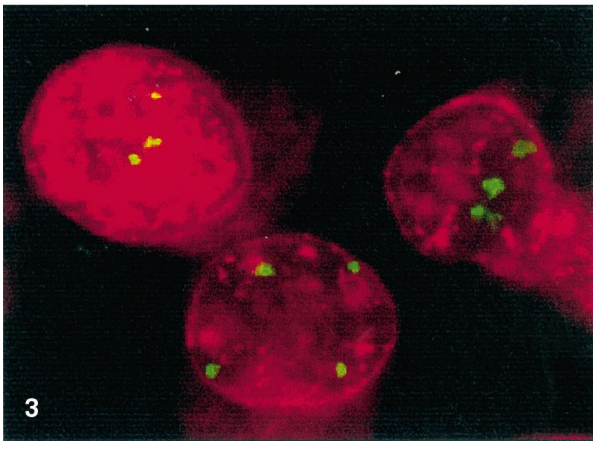
Results

Control FISH study

FISH on normal chromosome preparations showed that the YAC 19I11F probe was nonchimeric and hybridized specifically to 6q25.1 [16]. On the basis of extensive control studies on phytohemagglutinin-stimulated peripheral lymphocyte cultures from five healthy donors, the cut-off limit for detecting 6q deletions was set at 10%, which was above the highest cut-off level calculated as the mean of false-positive cells in control studies plus three standard deviations for any of the analyzed 6q YACs.







FICTION study

The results of the FISH study are given in Table 1. In 5 cell lines, more than 98% of cells had consistent copy numbers of chromosome 6, indicating that they contained homogeneous cell populations. CAL51 carried two, MCF-7 three, BRC230 and R103 four, and T-47D six copies of chromosome 6. In MDA-MB-231 there were two cell populations, one with disomy 6 and one with trisomy 6.

Deletions of at least one copy of the ESR gene were detected in four of the six cell lines. In BCR230 and T-47D, containing four and six chromosomes 6, respectively, different cell proportions with deletions of one or more copies of the ESR gene coexisted (Fig. 1). In R103, a small proportion of cells (20%) contained a deletion of the ESR gene. In MDA-MB-231, deletions of the ESR gene were found only in cells with trisomy 6, and not in cells with disomy 6. Thus, deletions of the ESR gene seem to be secondary changes in this cell line.

Combined immunophenotyping and FISH study

Comparing ER expression patterns and deletions of the ESR gene directly by means of the FICTION technique, no consistent relationship was observed. Two cell lines, MCF-7 and T-47D, were found to be ER expression positive. The other four cell lines were negative (Table 1). Of four cell lines with deletions of the ESR gene, three (BCR230, MDA-MB-231, R103) did not express ER, while T-47D did. Of two cell lines without a deletion, one was ER positive and the other, negative. In MDA-MB-231 and R103, deletions of the ESR gene were detected in 41% and 20% of cells, respectively, but hardly any cells expressed the ER. However, in T-47D with deletions of the ESR gene in all cells, 92% of cells expressed the ER. In MCF-7 and

Table 1 Results of combined immunophenotyping and fluorescence in situ hybridization (FISH) studies on breast carcinoma cell lines (– cells had no fluorescence or showed some dimly red point fluorescence in nuclei, + cells showed clear red but not intensive fluorescence in nuclei, ++ cells showed strongly distinct red staining with intensive pattern)

Cell lines	Dual-color FISH study				Combined immunophenotyping and FISH study					Status of ER expression
	No. of cells analyzed	Signals of D6Z1(%)	Signals of 19I11F(%)	Deletions of the ESR gene	No. of cells analyzed	Signals of 19I11F	Cells at ER expression level(%)			
							++	+	–	
CAL51	100	2 (100)	2 (97)	Not deleted	200	2		10 (5)	190 (95)	Negative
MCF-7	100	3 (100)	3 (98)	Not deleted	200	3	160 (80)	20 (10)	20 (10)	**Positive**
BRC230	100	4 (100)	2 (89)	**Deleted**	200	2		20 (10)	180 (90)	Negative
			3 (11)	**Deleted**	200	3		28 (14)	172 (86)	Negative
MDA-MB-231	293	2 (59)	2 (59)	Not deleted	200	2		20 (10)	180 (90)	Negative
		3 (41)	2 (41)	**Deleted**						
R103	146	4 (100)	3 (20)	**Deleted**	100	3		5 (5)	95 (95)	Negative
			4 (80)	Not deleted	200	4		4 (2)	196 (98)	Negative
T-47D	100	6 (99)	5 (19)	**Deleted**	100	5	62 (62)	22 (22)	16 (16)	**Positive**
			4 (68)	**Deleted**	200	4	140 (70)	50 (25)	10 (5)	**Positive**
			3 (13)	**Deleted**	100	3	65 (65)	28 (28)	7 (7)	**Positive**

◀ **Fig. 1** Dual-color fluorescence in situ hybridization (FISH) study on breast carcinoma cell line BRC230. A biotinylated YAC (yeast artificial chromosome)19I11F probe containing the ESR gene (estrogen receptor gene) locus visualized by Cy3 (*red*) and a digoxigenin-labeled D6Z1 probe specific for the centromere of chromosome 6 visualized by fluorescein isothiocyanate (FITC) (*green*) were used. Cells show four green hybridization signals for D6Z1 but three signals for the YAC probe, indicating a deletion of the ESR gene

Fig. 2 Combined immunophenotyping and FISH study on breast carcinoma cell line MCF-7 containing three copies of chromosome 6. Monoclonal mouse anti-human ER was visualized by Cy3 (*red*), and the YAC 19I11F probe was detected by FITC (*green*). Two cells show three hybridization signals for the YAC probe and thus contain no deletions of the ESR gene. One cell expresses the ER and the other does not

Fig. 3 Combined immunophenotyping and FISH study on breast carcinoma cell line T-47D with six copies of chromosome 6. Monoclonal mouse anti-human ER was visualized by Cy3 (*red*), and the YAC 19I11F probe was detected by FITC (*green*). Cells show three and four hybridization signals for the YAC probe. There was no consistent relationship between ER expression intensity and the copy numbers of the ESR gene

CAL51 90% and 95% of cells were ER positive and negative, respectively. In BRC230, which had a deletion of the ESR gene in all cells, 88% of cells were ER negative.

Direct comparison of ER expression with deletions of the ESR gene at the single cell level confirmed these results. Deletions of the ESR gene were detected both in ER-positive and ER-negative cells (Table 1). There were ER-negative cells without deletions and ER-positive cells with deletions (Fig. 2). Moreover, there was no correlation between the intensity of ER expression and the copy number of the ESR gene. Cells with two regular copies and cells with more than three copies of the ESR gene showed similar ER expression intensity (Fig. 3).

Discussion

In this study, simultaneous fluorescence immunophenotyping and FISH to detect chromosomal deletions of a tumor suppressor region was successfully applied for the first time in solid tumors. It was demonstrated in a series of breast carcinoma cell lines by directly comparing ER expression status and deletions of the ESR gene at the single cell level. This technique allows simultaneous display of the immunophenotype and certain genotypic changes of single tumor cells and has been shown to be feasible and very useful in the study of hematological neoplasms [31, 32, 34]. For detection of ESR gene deletion, we assumed that differences in signal numbers, i. e. lower numbers of hybridization signals of the YAC 19I11F probe than of the D6Z1 probe, indicated a deletion of the ESR gene. Nevertheless, we cannot exclude the possibility that this signal constellation is due to a gain of the short arm or the proximal part of the long arm of chromosome 6 including the centromere. Using the criteria defined above, deletions of the ESR gene were detected by dual-color FISH in four of the six breast carcinoma cell lines studied. By LOH studies, deletions of the ESR gene were detected in 27–41% of native breast cancers [4, 20, 26, 35]. Deletions of 6q25.1, the location of the ESR gene, were revealed by chromosome analysis and by comparative genomic hybridization (CGH) studies in 11% and in 14% of breast cancers, respectively [12, 19, 22, 27]. Thus, FISH and LOH studies seem to be more sensitive than chromosome analysis or CGH studies for detecting deletions of the ESR genes.

Our study revealed no consistent relationship between absent or reduced ER expression and deletions of the ESR gene in breast carcinoma cell lines. There were ER expression-positive cell lines that indeed contained deletions of the ESR gene and ER expression-negative cell lines carrying a regular number of ESR genes. Moreover, deletions of the ESR gene occurred in both ER-positive and ER-negative cells. Cells with two regular copies and cells with more than two copies of the ESR gene showed similar ER expression intensity. Thus, a gene dosage effect attributable to deletions of the ESR gene seems not to be the major cause of negative ER expression.

Our results were in agreement with those of previous studies. In a series of 95 breast cancers, Magdelenat et al. [13] compared ER expression levels measured by a fluorimetric assay and deletions of 6q as detected by chromosome analysis. Major variations in ER expression were shown to be independent of the assumed copy numbers of the ESR gene. Nevertheless, the authors suggested that gene dosage effect had a secondary, but significant, additional role. Sauer et al. [24], who used immunocytochemistry assay and FISH with a centromeric probe for chromosome 6, found no correlation between ER positivity or the percentage of ER-positive nuclei and numerical aberrations of chromosome 6 in breast cancers. In LOH studies, most breast carcinomas with LOH at the ESR gene locus were ER expression positive. No significant relationship between LOH at the ESR gene locus and ER expression status was observed [1, 4, 5, 7, 11]. Moreover, the amplification level of the ESR gene was found not to be associated with the level of ER expression [18]. Nevertheless, none of these studies directly compared ER expression status and deletions of the ESR gene at the single cell level.

So far, the mechanisms which control and regulate ER expression in breast carcinoma have not been elucidated. Alterations at the genomic, transcriptional or post-transcriptional level and changes in regulatory units of the ESR gene may be responsible for loss of ER expression. Several studies failed to show recurrent mutations, large genomic deletions, rearrangements or gene amplification of the ESR gene in breast cancer [23]. However, extensive methylation of the CpG island in the 5’ promoter region of the ESR gene is associated with loss of ER expression in breast cancer [21]. A number of variations of the ESR upstream regulatory region were identified from the MCF-7 and MDA-MB-231 breast cancer cell lines, and some of them were found to be associated with reduced ER expression [25].

Moreover, alternative splicing of the ESR gene is apparently a frequent event in breast tumors and may be involved in the silencing of ER expression. Several variant ESR mRNAs containing deletions of different exons, which might result from alternative splicing or from genomic mutations, were reported in BT-20, MCF-7, and T-47D [3, 8, 33]. Most of these variants lack hormone-binding domains and are expected to behave as dominant-negative effectors of wild-type ER [8, 28]. The variants may also result in truncated proteins, which are possibly not recognized by antibodies used in histochemical studies. Therefore, in addition to microscopically visible deletions, variations of the ESR upstream regulatory region and aberrant splicing might account for clinically relevant lack of ER expression.

In summary, we have demonstrated for the first time the feasibility of simultaneous fluorescence immunophenotyping and FISH to detect chromosomal deletions on solid tumor cells by directly comparing ER expression status and deletions of the ESR gene at the single cell level in a series of breast carcinoma cell lines and found no consistent correlation between absent or reduced ER expression and deletions of the ESR gene. Deletions of the ESR gene do not seem to be the major cause of ER expression negativity.

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