Macro-environment of breast carcinoma: frequent genetic alterations in the normal appearing skins of patients with breast cancer

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Genetic abnormalities in microenvironmental tissues with subsequent alterations of reciprocal interactions between epithelial and mesenchymal cells play a key role in the breast carcinogenesis. Although a few reports have demonstrated abnormal fibroblastic functions in normal-appearing fibroblasts taken from the skins of breast cancer patients, the genetic basis of this phenomenon and its implication for carcinogenesis are unexplored. We analyzed 12 mastectomy specimens showing invasive ductal carcinomas. In each case, morphologically normal epidermis and dermis, carcinoma, normal stroma close to carcinoma, and stroma at a distant from carcinoma were microdissected. Metastatic-free lymphatic tissues from lymph nodes served as a control. Using PCR, DNA extracts were examined with 11 microsatellite markers known for a high frequency of allelic imbalances in breast cancer. Losses of heterozygosity and/or microsatellite instability were detected in 83% of the skin samples occurring either concurrently with or independently from the cancerous tissues. In 80% of these cases at least one microsatellite marker displayed loss of heterozygosity or microsatellite instability in the skin, which was absent in carcinoma. A total of 41% of samples showed alterations of certain loci observed exclusively in the carcinoma but not in the skin compartments. Our study suggests that breast cancer is not just a localized genetic disorder, but rather part of a larger field of genetic alterations/instabilities affecting multiple cell populations in the organ with various cellular elements, ultimately contributing to the manifestation of the more 'localized' carcinoma. These data indicate that more global assessment of tumor micro- and macro-environment is crucial for our understanding of breast carcinogenesis.

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Recent studies have demonstrated genetic alterations in the normal epithelial and mesenchymal tissues surrounding breast carcinomas.^{1–5} These data strongly suggest that genetic abnormalities in microenvironmental tissues with subsequent alterations of reciprocal interactions between epithelial and stromal cells play a key role in the tumorigenesis of breast cancer. In our previous study, we showed that loss of heterozygosity (LOH) frequently occurs in the tumor-free and normal-appearing epithelial and mesenchymal tissue components

close to and away (at least 15-mm distance) from the breast cancer tissues.¹ Although most cases (73%) in our previous study revealed at least one identical LOH in both epithelial and stromal cells, several cases were associated with loss of certain microsatellite loci exclusively in the stroma suggesting that certain genetic alterations in the mammary stroma may even precede genotypic changes in the cancerous cells. On the other hand, the assessment of breast specimens (reduction mammoplasty) from women without breast disease in our previous study has failed to show any genetic alteration in either the stroma or the epithelial tissue component, demonstrating that genetic abnormalities can exclusively be identified in breasts with pathologic proliferations or changes.¹

Using cell cultures, a few reports have shown abnormal fibroblastic functions in morphologically

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normal-appearing fibroblasts taken from the skins of patients with breast carcinoma.^{6–9} Indeed, abnormal properties of skin fibroblasts displaying various oncofetal characteristics such as invasion of embryonic organ culture and increase of saturation densities in overcrowded culture conditions were demonstrated in 90% of patients with familial breast cancer and in 50% of the clinically unaffected first-degree relatives of patients suffering from familial breast cancer.^{6–8}

The genetic basis of this phenomenon, however, has not yet been explored. In this report, we present the first data concerning the frequent occurrence of LOH and microsatellite instability (MSI) in the normal skin (epidermis/dermis) of breast cancer patients. The frequent genetic alterations in morphologically normal epidermis and dermis raise the intriguing possibility that genetic changes of macro-environmental tissues (tissues at a large distance to breast cancer) may also play a significant role in the breast carcinogenesis.

Materials and methods

Tissue Sample Preparation

Samples (mastectomy specimens) from 12 female patients containing invasive ductal breast carcinomas were selected from the files of the Department of Pathology, Medical University of Graz. All cases were accompanied with metastatic-free axillary lymph nodes that served as normal controls. None of the cases was associated with a history of radiation therapy, chemotherapy, a positive family history for breast cancer, or any hereditary cancer. In each case, morphologically normal epidermis, dermis, invasive carcinoma, non-invasive (*in situ*) carcinoma, normal stroma close to carcinoma, and stroma at a distant (at least 30 mm) from invasive carcinoma were microdissected. All skin samples were macroscopically and histologically tumor free. Samples from the skins were not associated with inflammation or any histologically recognizable degenerative change. Epithelial cells from

morphologically clear-cut normal ducts and ductules (acini) at a distant (at least 30 mm) and lymphatic tissue from lymph nodes were also microdissected. The tissue microdissections of different areas were performed as described previously.¹

Briefly, precise microdissection of selected areas from formalin-fixed, paraffin-embedded tissue was performed by one experienced pathologist (FM) under direct light microscopic visualization. Hematoxylin-stained sections were microdissected manually with a disposable, sterile, 30-gauge needle.

Samples from 10 women with bilateral reduction mammoplasty (RM) were included in the study and served as an 'external control' for normal epithelium and stroma unassociated with any neoplastic process; these women did not show any clinical, radiological, or histomorphological abnormalities in their breasts. In each RM case, the morphologically normal ductal epithelium, the intervening normal stroma, and the skin were microdissected and analyzed for possible LOH or MSI.

Microsatellite Markers

To avoid artifactual patterns (LOH or MSI artifacts), a minimum of 10 ng template DNA content was required before performing PCR amplification. Using PCR, we examined DNA extracts from the microdissected tissues with 11 microsatellite markers on chromosomes 3p, 10q, 11q, 16q, 17p, and 17q which are known for a high frequency of allelic imbalances in breast cancer.¹ The sequences of these microsatellites were obtained from the 'Genome Data Base' (<http://www.gdb.org>). The examined polymorphic (microsatellite) markers used in our study are shown in Table 1.

Sample Analysis

Tissue sections were cut at 5 μ m on a MICROM HM 355S microtome (Carl Zeiss, Jena, Germany),

Table 1 Primer sequences and annealing temperatures for polymorphic (microsatellite) markers used in this study

Marker	Chrom. location	Sense primer	Antisense primer	Ann. T.
D3S1067	3p21.1–3p14.3	TCATCTATCTCCCAACTGTTGAG	GAGCACTACCTGTTTAAGATAGG	61°C
D3S1300	3p21.1–3p14.2	AGCTCACATTCTAGTCAGCCT	GCCAATTCCTCCAGATG	51°C
D10S541	10q22–10q23	AAGCAAGTGAAGTCTTAGAACCACC	GCCACAAGTAACAGAAAGCCTGTCTC	61°C
D10S2491	10q23–10q23	GTTAGATAGAGTACCTGCACTC	GTTAGATAGAGTACCTGCACTC	61°C
D10S2492	10q23–10q23	TGCAGTGAGCTGTGAAGATG	GTGTTTCTCTTACTACCTATGTGA	56°C
D11S1311	11q21–11q23.2	TGTTGACTAAATGAGTGTCAGACCA	GCCTTGAGGGCAGGAATAGTGTC	59°C
D16S402	16q24.2–16q24.2	TTTTGTAACCATGTACCCCC	GATTATAGGGCCATGACCAG	52°C
D16S518	16q23.1–16q24.2	GGCCTTTTGGCAGTCA	GACCTTGGCCTCCCAACC	61°C
D17S579	17pter–17qter	AGTCCTGTAGACAAAACCTG	GCAGTTTCATACCAAGTTCCT	61°C
D17S785	17pter–17qter	ATCCCTGGAGAGTGAAAATG	GAAGGCCAACCTGAAAATAA	60°C
Tp53.15	17p13.1–17p13.1	AGGGATACTATTACGCCCGAGGTG	GACTGCCACTCCTTGCCCCATTG	60°C

Ann. T., annealing temperature; Chrom. location, chromosomal location of indicated marker; ter, terminal part of chromosome.

deparaffinized in xylene and stained with methylene blue. Microdissected tissue samples were incubated in xylene for 30 min, spun in a microcentrifuge and washed first with absolute ethanol and subsequently with 70% ethanol. The pellets were dried in a vacuum centrifuge at room temperature, resuspended in 180 μ l of 50 mM Tris-buffer (pH 9.0) and incubated with 500 ng proteinase K at 37°C for 24 h. After the incubation all samples were purified with the Qiagen DNA-purification Kit (Qiagen, Vienna, Austria) in accordance to the manual.

The microsatellites loci were amplified by PCR in a total volume of 20 μ l. The reaction mixture contained 2 μ l of the 10 $\times$ PCR-buffer, 2 nM each of dATP, dCTP, dGTP, and dTTP, 5 pM of both sense and antisense primer, 50 ng of template DNA and 0.5 U of the Qiagen Hotstart Polymerase. All sense primers were labeled with D2, D3, and D4 at their 5'-end (Beckman Coulter, CA, USA).

After one initial cycle at 92°C for 12 min, 40 cycles were performed under following conditions: 92°C, 1 min; 51–61°C (depending on the particular primer set; see Table 1 for details), 1 min; 72°C, 1 min; followed by a final extension at 72°C for 7 min. A small amount of the PCR products were mixed with 0.5 μ l CEQ™ D1-labeled DNA size standard-400 (Beckman Coulter) and with 40 μ l CEQ™ sample loading solution (Beckman Coulter). These mixtures were separated on the capillary-sequencer Beckman Ceq 8000 (Beckman Coulter) in accordance to the manual.

LOH was defined as complete absence or at least 70% reduction of one allele as assessed by direct visualization. MSI was defined as the presence of additional bands (peaks) or abnormal shifting of bands (peaks) compared to normal lymphatic tissue of axillary node. To evaluate the reproducibility of the results showing LOH or MSI, the assay was repeated at least two times on each sample under the same condition. Moreover, all cases with genetic abnormalities (LOH or MSI) were microdissected again to obtain DNA for independent PCR analyses. In all cases, results identical to the original reactions were observed, demonstrating the fidelity of the methods.

Results

Seven cases (58%) showed genetic alterations (either LOH or MSI) in both their skins (epidermis and dermis) and cancerous (invasive and non-invasive) tissues using at least one DNA marker (Table 2). Ten cases (83%) revealed genetic changes in the skin tissues (epidermis and/or dermis). In 8 of these 10 cases (80%), at least 1 microsatellite marker displayed LOH or MSI in the skin tissue components which was absent in carcinoma. On the other hand, five cases (41%) showed alterations of certain

Table 2 Summary of genetic alterations occurring in both tumor and non-tumorous tissues

Case	Molecular marker										
	D3S1067	D3S1300	D10S541	D10S2491	D10S2492	D11S1311	D16S402	D16S518	D17S579	D17S785	TP53.15
1	—	LOH in ICA, D	—	—	—	—	—	—	LOH in ICA, SCICA, NS	LOH in NS	—
2	LOH in SCICA, NE	NI	LOH in ED, D, NE, SCICA	—	—	—	—	—	—	—	—
3	LOH in ED	—	LOH in DCIS, ICA, SCICA, D, ED	—	—	LOH in ICA	LOH in DCIS, D, ED	—	—	LOH in NE, D, ED, SCICA	MSI in DCIS
4	—	—	—	—	MSI in SCICA	—	—	—	—	—	NI
5	—	LOH in ICA, ED, NS	—	—	LOH in D, NS	LOH in ICA	—	—	LOH in ICA	LOH in D, ED	—
6	—	—	LOH in D, ED	—	—	—	—	—	—	—	—
7	—	—	—	—	—	—	—	—	—	—	—
8	—	—	—	—	LOH in D	—	—	—	—	—	—
9	LOH in ED, NS	—	—	—	—	—	MSI in DCIS, D, ED	—	—	—	—
10	—	LOH in ICA	—	MSI in ED	MSI in SCICA, NS, ED	LOH in ICA, DCIS	—	—	LOH in NS	—	—
11	—	LOH in D	—	—	LOH in DCIS, D	—	—	—	—	—	—
12	—	LOH in SCICA, NS	MSI in ED	MSI in ICA, NS	—	—	—	NI	—	LOH in D, ICA	LOH in ICA

D, dermis; DCIS, ductal carcinoma *in situ*; ED, epidermis; ICA, invasive carcinoma; LOH, loss of heterozygosity; MSI, microsatellite instability; NE, normal epithelium (at a distant from cancer); NI, non-informative; NS, normal stroma (at a distant from cancer); SCICA, stroma close to invasive carcinoma.

Table 3 Genetic alterations (LOH and MSI) in cancerous and non-cancerous breast tissues

Case	Invasive carcinoma	Ductal carcinoma in situ	Normal epithelium (at a distant from cancer)	Stroma close to invasive carcinoma	Normal stroma (at a distant from cancer)	Epidermis	Dermis
1	●	—	—	—	●	—	●
2	●	—	●	●	●	●	●
3	●	●	●	●	—	●	●
4	—	—	—	◆	—	—	—
5	●	—	—	—	●	●	●
6	—	—	—	—	—	●	●
7	—	—	—	—	—	—	—
8	—	—	—	—	—	—	●
9	—	◆	—	—	●	●	● ◆
10	●	●	—	◆	● ◆	◆	—
11	—	●	—	—	—	—	●
12	● ◆	—	—	●	● ◆	◆	●

●, LOH (loss of heterozygosity); ◆, MSI (microsatellite instability); —, no change.
All changes are presented with at least one polymorphic DNA marker.

loci observed exclusively in the carcinoma but not in the skin compartments (Tables 2 and 3).

More interestingly, the comparison of genetic changes in the epidermal and dermal tissues showed concurrent and independent alterations. While three cases revealed concurrent genetic changes in the epidermis and dermal tissues with at least one DNA marker, six cases displayed alterations in dermis but not in their epidermis. Three cases also revealed changes in their epidermis but not in the dermis. Moreover, 6 out of 12 cases revealed LOH (with at least one polymorphic marker) in normal stromal tissues at a distant (at least 30 mm) from breast cancer. A general comparison of obtained LOH and MSI revealed a total of 53 LOH and 12 MSI in different tissue components of 12 cases. With regard to the polymorphic DNA markers, 9 out of 11 showed at least one LOH whereas five markers revealed at least one MSI. The DNA markers with a high frequency of LOH were *D3S1300* (10 × LOH in five cases), *D10S541* (11 × LOH in three cases), *D17S785* (9 × LOH in four cases), *D17S579* (5 × LOH in three cases), *D10S2492* (5 × LOH in three cases), *D3S1067* (5 × LOH in three cases), and *D11S1311* (4 × LOH in three cases). The microsatellite markers associated with the most frequent MSI were *D10S2491* (3 × MSI in two cases), *D10S2492* (4 × MSI in two cases), and *D16S402* (3 × MSI in one case). Table 2 summarizes the distribution of LOH and MSI among the examined cases. Examples of LOH and MSI are demonstrated in Figure 1. In contrast to the cases with breast cancer, not a single case in the control RM group (bilateral RM specimens) revealed LOH or MSI in either its epithelial or stromal components.

Discussion

It is increasingly becoming clear that mammary stroma and tumor microenvironment play a key role

in inducing neoplastic transformation of epithelial cells, recapitulating its role in normal mammary duct development.^{10–13} Our group was the first that recognized concurrent and independent genetic alterations (LOH) in the stromal and epithelial cells of mammary carcinoma and raised the intriguing possibility that the mammary stroma in breast cancer may represent a neoplastic rather than a reactive response to the carcinoma.¹ Since publication of our previous observation, several other groups have confirmed the occurrence of genetic abnormalities in the normal-appearing stromal cells close to the breast carcinomas.^{2–5}

In the current study, we provide, for the first time, evidence of common genetic alterations in the tumor-free and morphologically normal-appearing mammary skin of the patients with breast cancer. These data have implications for understanding of tumorigenesis in general and of breast cancer in particular. Our findings support the concept of micro- and macro-environmental genetic changes in breast tissues where carcinomas develop.^{10–13}

Although the results of our present study are ‘unexpected’ or even may be viewed as ‘unmatchable’ to the current theory of carcinogenesis, we believe that, like our first report on genetic changes in the stromal cells of breast carcinoma, this study deserves serious consideration. One needs to keep in mind that genetic instability of various cell types in the whole breast allows numerous genetic and epigenetic alterations to accumulate during carcinogenesis without significantly changing phenotype until they are qualitatively and/or quantitatively sufficient to be selectively advantageous in the tumor environment. Exposure of the breast to a variety of carcinogenetic and environmental stress agents may contribute to cancer pathogenesis by (a) induction of mutations in epithelial cells, (b) induction of genetic alterations in the stromal cells, (c) induction of a variety of growth factors with subsequent abnormal signal transduction, and (d) alterations of extracellular matrix (ECM).

Figure 1 Different genetic alterations in cancerous and non-cancerous breast tissues. (a) LOH in invasive carcinoma (ICA) using polymorphic marker *D17S579* in case no. 5. (b) Using polymorphic marker *D10S541* in case no. 2, LOH was identified in epidermis (ED) and dermis (D), but not in invasive carcinoma (ICA). (c) Case no. 12 with MSI in epidermis (ED) using marker *D10S541*. (d) LOH identified in dermis (D) of case no. 11 using polymorphic marker *D10S2492*.

Numerous growth factors such as fibroblastic growth factors, transforming growth factors (TGF- α and - β family), stem cell factor, and hepatocyte growth factor (HGF) are known to contribute to the morphogenic and mitogenic functions of stromal cells which are involved in both physiologic (ie wound healing) and neoplastic processes.^{14–17} It is well known that skin fibroblasts (dermis) and epidermal epithelial cells produce several of these growth factors. Recently, HGF, which is mainly produced by fibroblasts, has been identified as one of the most potent mitogenic factors for proliferation

of epithelial cells in a variety of organs.^{14–15} Genetic alterations with loss of tumor suppressor genes (TSGs) in the stromal cells may lead to subsequent abnormal production of growth factors with constant signal transduction in the epithelial cells. Indeed, a recent study has demonstrated that stromal cells have a significant impact on the carcinogenic process in adjacent epithelial through TGF- β and activation of paracrine HGF signaling. With regard to abnormal migratory behavior of skin fibroblasts of breast cancer patients, a recent study has revealed that this behavior is related to

migration-stimulating factor (MSF), which is produced by both fetal and cancer patient fibroblasts, but not by adult fibroblasts.¹⁸ Interestingly, it has been shown that MSF is expressed by fetal skin keratinocytes, breast carcinoma cells, and tumor-associated vascular endothelial cells.¹⁸ In addition, there is evidence that apart from stimulating cell migration, MSF is a potent stimulator of angiogenesis.¹⁸

It is of note that the polymorphic markers with a high frequency of LOH in this study represent parts of several putative TSGs. For example, the polymorphic locus *D13S1300* (3p14.2) is within the *FHIT* gene. The loss of *FHIT* gene has been documented as one of the earliest genetic abnormalities in several malignant neoplasms including breast carcinoma, osteosarcoma, and Ewing sarcoma.^{19–20} The polymorphic locus *17S785* (17q24-25) is harbored in several potential TSGs that are frequently lost in alveolar soft-part sarcoma, dermatofibrosarcoma protuberance, and fibrosarcoma in patients with von Recklinghausen's neurofibromatosis as well as breast carcinoma.^{21–22} The cytogenic locus 17q21 (*D17S579*) is located in the *BRCA1* gene, a TSG that is commonly lost in the familial breast and ovarian carcinomas.²³ The cytogenic locus 11q-21–23.2 (*D11S1311*) is distal and close to the ataxia teleangiectasia gene, another potential TSG which is commonly lost in breast carcinoma.²⁴

Several studies have clearly shown that in addition to growth factors and hormones, ECM that surrounds tissues also contains signaling molecules that are responsible for maintenance of tissue form and function and that ECM plays an important and dynamic role in carcinogenesis.^{25–27} Therefore, genetic abnormalities in the skin stroma may significantly change the physiological composition of the ECM with subsequent alteration in the reciprocal epithelial–ECM interaction(s).

The presence of genetic alterations in the skin of patients with breast cancer and the lack of any genetic abnormality in RM specimens from women without breast disease are in concordance with reports that have shown abnormal fibroblastic functions in morphologically normal-appearing fibroblasts in the skin of patients with breast carcinoma. Indeed, abnormal skin fibroblasts displaying various oncofetal characteristics were demonstrated in 90% of patients with familial breast cancer and 50% of the clinically unaffected first-degree relatives of patients suffering from familial breast cancer.^{6–9} Moreover, abnormal skin fibroblasts with a high level of enhanced reactivation of herpes simplex virus have been found in a variety of hereditary cancer-prone syndromes such as retinoblastoma, polyposis coli, neurofibromatosis type 1 and 2, dysplastic nevus syndrome, von Hippel–Lindau syndrome, and multiple endocrine neoplasia type 2, suggesting that loss of one allele of putative TSGs may activate cellular processes that result in the induction of the enhanced reactivation

response and that functionally abnormal skin fibroblasts may be related to the process of carcinogenesis.²⁸ The frequent genetic alterations in the skin, particularly LOH near some of the putative TSGs, as identified in our study, may partly explain some of the abnormal fibroblastic functions that have been observed in the skin of patients with breast cancer or some of the cancer-associated hereditary diseases.²⁸ It is, however, important to point out that none of the cases examined in the present study had a history of familial breast cancer or any cancer-associated hereditary disease.

On the basis of the gene expression profiles of fibroblasts from 10 anatomic sites, Chang *et al*,²⁹ were recently able to identify a stereotyped gene expression program in response to serum exposure that seems to reflect the multifaceted role of fibroblasts in wound healing. Recently, it has been shown that genes induced in the fibroblast serum-response program are expressed in tumors by the tumor cells themselves, by tumor-associated fibroblasts, or both. Moreover, the molecular features that define this wound-like phenotype are evident at an early cancer and predict increased likelihood of metastasis and death in breast, lung, and gastric cancer.²⁹ In a subsequent study of 295 early breast carcinomas, Chang *et al*³⁰ showed that both overall survival and distant metastasis-free survival are significantly diminished in patients whose carcinomas expressed this 'wound-response signature' compared to tumors that did not express this signature.³⁰ At this point, it is not clear whether the observed genetic changes in our current study are related to breast cancer initiation or are acquired 'reactive' genetic alterations which are more associated with tumor progression.

Our data suggest that breast cancer is not just a localized disordered genetic process, but rather part of a larger field of genetic alterations/instabilities affecting multiple cell populations in the organ with various cellular elements ultimately contributing to the manifestation of the more 'localized' carcinoma. While a field of somatic genetic alterations may precede or contribute to the manifestation, survival, and progression of breast cancer, the presence of genetic instability in the whole organ may play a key role in the development of a 'clonal expansion' of epithelial cells with abnormal differentiation or loss of normal growth control. Our data raise the intriguing possibility that genetic alterations in macro-environmental epithelial and mesenchymal tissues, or tissues far away from cancer, may represent an integral part of breast carcinogenesis. Furthermore, these data challenge the current somatic mutation theory of tumorigenesis, a prevailing paradigm in cancer research, which focuses on the accumulation of genetic changes (mutations) in a single cell progressing to a malignant phenotype.^{31–32} Our results are rather in favor of the 'tissue organization field theory' which considers neoplasia as the default state of several

cell types resulting in alteration and disruption of tissue structures with subsequent abnormal interactions among various epithelial and mesenchymal cells within the involved organ.^{33–37}

The high rate of genetic changes in the normal-appearing mammary skin in breast cancer patients, as identified in our study, and frequent genetic alterations in morphologically normal epithelial and stromal cells, as described previously,¹ clearly demonstrate that 'normal' breast tissue, even far away from the cancerous tissue, should not be used as a normal control for genetic studies. Tumor-free tissue outside of the breast (for example metastatic-free axillary lymph nodes) may be better candidates to serve as normal cell population for genetic studies of breast cancer.

One of the limitations of our current study is the small sample size that does not allow a meaningful statistical analysis of the results. Nevertheless, we believe that the results of our study, albeit using a small number of breast cancer cases, can serve to generate an alternative hypothesis in breast carcinogenesis, which certainly needs to be confirmed by molecular analysis of a large number of cases. Another limitation of our study is its descriptive nature. We, however, would like to emphasize that the current study should merely be regarded as a first step in the recognition of molecular changes in the skin of breast cancer patients or in macro-environmental breast tissue. Further studies using more complex molecular techniques applied in both *in vitro* and *in vivo* systems are needed to better understand the observed genetic alterations in the skins of patients with breast cancer.

In conclusion, our study shows that genetic alterations in the normal-appearing mammary skins of patients with breast cancer are common. Rather than focusing purely on the carcinoma cells, a more global assessment of tumor micro- and macro-environment is crucial for our understanding of development and progression of cancer in general and breast cancer in particular. This could furthermore alter current therapeutic approaches to breast carcinoma.

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Disclosure/conflict of interest

All authors declare that there is no conflict of interest.

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