Pathology - Research and Practice 215 (2019) 152634

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Negative prognostic value of intra-ductal fat infiltrate in breast cancer

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ARTICLE INFO

Keywords:
Breast cancer
Intra-ductal fat infiltrate
Estrogen
Microcalcifications

ABSTRACT

Background: Recent studies showed a correlation between Body Mass Index and both breast cancer occurrence and progression. Nevertheless, no study reported an accurate evaluation of intra-ductal fat infiltrate. Therefore, the main aim of this study was to evaluate the putative association between intra-ductal fat infiltrate (IDFi) and breast cancer subtypes by using digital pathology.

Methods: We retrospectively collected 220 breast biopsies. Paraffin serial sections were used for haematoxylin and eosin staining and immunohistochemical evaluation of the following markers: estrogen receptor (ER), progesterone receptor (PR), Ki67 and c-erb2. Three haematoxylin and eosin sections for each paraffin block were digitalized. Digital slides were used to evaluate the areas of IDFi. Five randomized areas were evaluated for each slide. By using GraphPad software IDFi areas was correlated with a) breast cancer histotype, b) presence of microcalcifications and c) biomarkers expression.

Results: Breast biopsies were classified as follow: 20 normal breast, 50 benign lesions, and 150 malignant lesions (85 ductal *in situ* carcinomas; 65 ductal infiltrating carcinomas). Statistical analysis showed a significant increase of IDFi in malignant lesions as compared to both normal breast and benign lesions. We noted higher IDFi in breast ductal carcinomas as compared to lobular lesions. Significant differences were observed between breast lesions with microcalcifications respect to lesions without calcifications. Noteworthy, we also found a positive association between IDFi and the expression of both ER and Ki67.

Conclusion: Results of our study highlighted the possible role of fat in breast cancer progression suggesting a negative prognostic value of IDFi.

1. Introduction

According to the most recent epidemiological data, breast cancer shows the highest incidence representing the second leading cause of death in women [1,2]. In detail, about 1 out of 8 women in the USA develops an invasive breast cancer lesion during their lifetime [1]. Cancer progression and metastasis are the main events related to poor survival in breast cancer patients. Reduction of the lifetime span up to the 10% [1].

The prognosis for breast cancer depends on the biochemical profile of the tumor itself as well as on its stadiation, which determines the possibility of conducting a normal life against a lifetime span reduction up to 10% [3]. The biochemical information are obtained through an histopathological and immunohistochemical study of the sample, which is usually formed by the primitive lesion and axillary lymph nodes [3]. The TNM system (Tumor, Nodes, Metastasis) uses as main prognostic factors 1) discrimination whether the carcinoma is infiltrating or *in situ*, 2) tumor's dimension 3) presence of metastasis, 4) axillary lymph nodes status [4]. The molecular subtypes have been identified through the analysis of the estrogen receptor (ER), the progesterone receptor (PR) and HER2 [5]. ER is usually evaluated through immunohistochemical analysis and its positivity correlates with a good prognosis [5]. Being a

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https://doi.org/10.1016/j.prp.2019.152634
Received 13 July 2019; Received in revised form 12 September 2019; Accepted 12 September 2019

nuclear transcriptional factor, it stimulates cellular proliferation after binding with its substrate. Her 2/neu is a gene located on the chromosome 17, which codifies for EGFR2, which in turn stimulates cancer growth [5]. Her2 neu is mainly used as a marker for tumor aggressiveness. The proliferation is also evaluated through Ki67 and it is used both as a prognostic and a predictive factor [5].

Growing evidence from both clinical and preclinical studies indicate that adiposity is associated with breast cancer risk and may act as a negative prognostic factor and therefore influence breast cancer recurrence and survival [6–9]. These observations have recently been supported in mechanistic studies, observing that adiposity-associated factors, such as hormones, lipids, adipokines, and pro-inflammatory mediators are associated with breast cancer development and progression [10,11]. Accumulating studies point to a role of mammary adipose tissue (MAT) adjacent to the tumors in breast cancer development and progression, as adipose tissue (AT) represents a major component of the breast tumor microenvironment [9–11].Nevertheless, to the best of our knowledge, no histological studies were performed about the association between the area of intra-ductal fat infiltrate (IDFi) and breast cancer characteristics.

Therefore, the main aim of this study was to evaluate the putative association between IDFi area and breast cancer histological subtypes by using digital pathology.

2. Material and methods

2.1. Breast samples collection

In this retrospective study, we enrolled 220 patients from which we collected one breast biopsy each. Our study protocol was approved by the "Policlinico Tor Vergata" independent ethical committee (reference number # 94.13). All experimental procedures were carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all patients prior to surgery. Specimens were handled and carried out in accordance with the approved guidelines.

From each biopsy, paraffin serial sections were obtained to perform histological classifications, immunohistochemical and digital pathology analysis.

2.2. Histology

After fixation in 10% buffered formalin for 24 h, breast tissues were paraffin embedded. Four μm thick sections were stained with hematoxylin and eosin (H & E) [12].

2.3. Immunohistochemistry

To better define the molecular profile of breast cancer, we employed immunohistochemical techniques to study the expression of ER, PR, Ki67 and HER2.

Briefly, sections were stained using the automatic Bench Mark system (Ventana, Tucson, AZ, USA). First, 3-μm thick sections were pretreated with a CC1 reagent (Ventana, Tucson, AZ, USA) and then incubated with primary monoclonal antibodies listed in Table 1. Reactions were revealed using an ultraView Universal DAB Detection Kit (Ventana, Tucson, AZ, USA). Immunohistochemistry was evaluated by two blind observers.

2.4. Evaluation of IDFi areas by digital pathology

H & E paraffin sections have been digitized by using the histological scanner iScan Coreo (Ventana, Roche). Each slide was digitalized at 40x magnifications. By using the software Image viewer IDFi areas were evaluated. Specifically, for each slide four areas were selected and used to discriminated the regions containing breast ducts (μm^2) from regions containing adipose tissue (μm^2) (until the single adipocyte).

The following criteria has been used to select breast areas:

- For normal breast and *in situ* lesions, each selected area included at least 8–10 ductal structures (fat inside interductal stroma).
- For infiltrating breast carcinomas, each selected area included at least 1 cm^2 of infiltrating lesions
- Only fat tissue interposed a) among the 8–10 ductal structures (benign/*in situ* lesions) or b) among infiltrating breast cancer cells has been evaluated.
- Fat tissue surrounding the ductal structures or infiltrating breast cancer cells has not been evaluated

The mean value of the ratio between breast ducts area and adipose tissue area was calculated. Results are expressed as the mean value of the fat area/duct area ratio.

2.5. Statistical analysis

By using GraphPad software IDFi areas was correlated with a) breast cancer histotype, b) presence of microcalcifications and c) biomarkers expression (a and b Mann Whitney test; c Pearson correlation).

3. Results

3.1. Histological analysis

According to Nottingham Histological system [13] breast biopsies were classified as follow: 20 normal breast (NB), 50 benign lesions (BL) (28 fibroadenomas and 22 fibrocystic mastopathy), 150 malignant lesions (85 ductal *in situ* carcinomas; 65 ductal infiltrating carcinomas). No lobular or special type breast carcinomas were found. Baseline characteristics are showed in Table 2.

3.2. Immunohistochemistry

HER2, ER, PR, and Ki-67 status were evaluated by immunohistochemical reaction in all malignant breast tissues. HER2 status was assessed according to ASCO-CAP 2013 guidelines [14] by a scoring system of 0–3. ER, PR, and Ki-67 were evaluated as percentage values of positive breast cancer cells. Results are reported in Table 3.

3.3. Evaluation of IDFi areas

Our analysis showed a significant group effect in the prevalence of IDFi area among NB, BL and ML groups ($p < 0.0001$) (Fig. 1A). Mann

Table 1
List of primary antibodies.

Antibody	Characteristics	Primary antibody	Dilution	Retrieval
anti-ER	Estrogen Receptor (ER) (SP1) Rabbit Monoclonal;Ventana, Tucson, AZ, USA	20 min	Pre-diluited	CC1 64 min
anti-PR	Progesterone Receptor (PR) (1E2) Rabbit Monoclonal; Ventana, Tucson, AZ, USA	16 min	Pre-diluited	CC1 64 min
anti-Ki67	Ki-67 (30-9) Rabbit Monoclonal Primary; Ventana, Tucson, AZ, USA	16 min	Pre-diluited	CC1 64 min
anti-HER2	HER-2/neu (4B5) Rabbit Monoclonal Primary; Ventana, Tucson, AZ, USA	12 min	Pre-diluited	CC1 36 min

Table 2
Baseline characteristics of patients.

	NB (n = 20)	In situ (n = 85)	G_1 (n = 19)	G_2 (n = 25)	G_3 (n = 21)
Age	49,23 ± 3,26	56,34 ± 2,12	58,80 ± 1,52	59,52 ± 1,16	61,00 ± 1,55
Menopause	12 (60,00%)	64 (75,29%)	14 (73.68%)	21 (84.00%)	13 (61,90%)
Age of Menopause	49,11 ± 4,13	49,52 ± 1,68	50,16 ± 2,03	49,98 ± 2,14	51,32 ± 1,03
BMI < 20	8 (40.00%)	22 (25,88%)	8 (42,10%)	6 (24,00%)	5 (23,81%)
BMI 20-30	7 (35,00%)	38 (44,70%)	7 (36,84%)	10 (40,00%)	14 (66,67%)
BMI > 30	5 (25,00%)	25 (29,42%)	4 (21,06%)	9 (36,00%)	2 (9,52%)
Children					
yes	14 (70,00%)	64 (75,29%)	14 (73,68%)	21 (84,00%)	17 (80,95%)
no	6 (30,00%)	21 (24,71%)	5 (26,32%)	4 (16,00%)	4 (19,05%)
Age at first Pregnancy	22,36 ± 2,38	24,25 ± 2,22	23,87 ± 3,65	21,39 ± 2,25	22,74 ± 1,89

Table 3
Evaluation of Breast Cancer Prognostic markers.

	Percentage of tumor cells positive						HER2 status			
	ER		PR		Ki67		score 0 n o staining or faint incomplete < 10% of tumor cells	score 1 f aint incomplete membrane staining > 10% of tumor cells	score 2 m embrane staining incomplete > 10%, or complete and intense < 10% of tumor cells	score 3 membrane staining complete, intense > 10 % of tumor cells OR FISH POSITIVE
	< 75	< 75	< 75	< 75	< 15	< 15				
In situ	70	15	63	22	78	7	21	42	19	3
Infiltrating	55	10	51	14	35	23	6	22	23	14

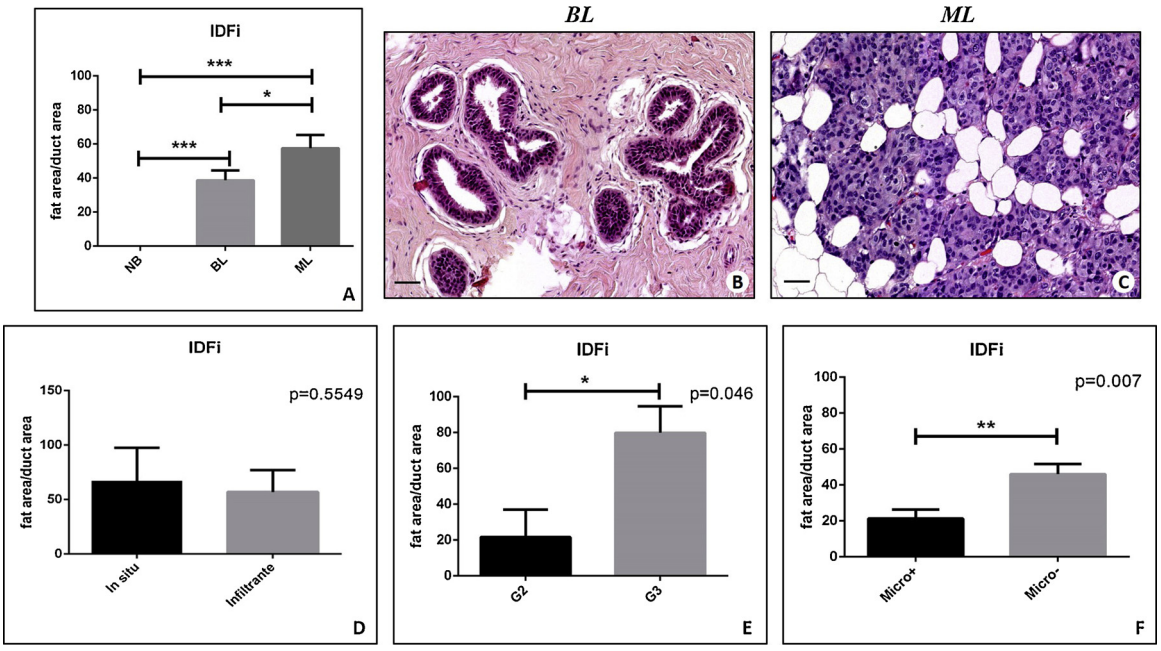

Fig. 1. Intra ductal fat infiltrate (IDFi) in breast tissues. A) Graph shows the ratio between IDFi area and ductal area in normal breast (NB), benign lesions (BL) and malignant lesions (ML). B) Image displays a benign breast lesion with no IDFi. C) Image shows an infiltrating breast carcinoma with high amount of IDFi. Scale bar represents 100 μm in all images. D) Graph shows no significant difference in IDFi between *in situ* and infiltrating breast malignant lesions. E) Graph displays IDFi in G2 and G3 breast infiltrating carcinomas. F) Graph shows IDFi in breast lesions with microcalcifications (micro +) and without macrocalcifications (micro-).

Table 4
Table shows Pearson analysis among breast cancer biomarkers and intra-ductal fat areas.

	ER	*PR*	*Ki-67*	*HER2*	*IDFi area*
ER		0,33518	0,02465381	0,7672254	0,01100477
PR	0,33518		0,1230531	0,3684826	0,286967
Ki67	0,02465381	0,1230531		0,3673654	0,006978354
HER2	0,7672254	0,3684826	0,3673654		0,576166
IDFi area	0,01100477	0,286967	0,006978354	0,576166	

Whitney test displayed a significant increase of IDFi area in ML (5933 ± 7.18) respect both BL (3868 ± 5761) and NB groups (0,003,244 ± 0,003,244) (NB *vs* BL $p < 0.0001$; NB *vs* ML $p < 0.0001$; BL *vs* ML $p = 0.038$) (Fig. 1A,B,C). No significant difference in IDFi area was observed between *in situ* (6714 ± 3027) and infiltrating breast carcinomas (5697 ± 20.01) (p = 05,569) (Fig. 1D). Also, analyzing the IDFi area among histological grade of infiltrating breast carcinomas, we found a significant increase of IDFi in G3 grade (2165 ± 1228) carcinomas respect to G2 one (7988 ± 1483) ($p = 0.046$) (Fig. 1E). Noteworthy, the presence of IDFi showed a positive correlation with the presence of breast microcalcifications (Fig. 1F). In particular, we found a significant increase of IDFi area in breast lesions (both BL and ML) with microcalcifications (micro+ 4604 ± 5683) as compared with lesions (both BL and ML) without microcalcifications (micro- 2128 ± 5077) ($p = 0.007$).

3.4. Correlation among breast cancer biomarkers and intra-ductal fat areas

To investigate the possible prognostic role of IDFi in breast cancer, we performed a linear correlation analysis (Pearson's Correlation) between IDFi and the expression of ER, PR, HER2 and KI67 (Table 4). Remarkable, we found a positive linear correlation between IDFi and ER expression (r^2 001,100,477) and between IDFi and Ki67 (r^2 0,006,978,354). A significant positive linear correlation was also found between Ki67 and ER (r^2 002,465,381).

4. Discussion

Breast Cancer is the most diagnosed non-skin cancer in women [1,2]. Over the last 20 years, improvements in both breast cancer diagnosis and treatment have led to a significant reduction of cancer-related mortality and an increase of patient quality of life [1]. Despite the recent introduction of new and promising genetic and molecular methods for the characterization of breast cancer, morphological classification continues routinely employed. In this context, estrogen receptor (ER), progesterone receptor (PR), Ki67 and Human epidermal growth factor receptor 2 (HER2) status are currently the only prognostic markers which can be considered predictive of therapy response [15–17]. Research based on gene expression profiling has been used to detect 4 breast cancer molecular subtypes, Luminal A, Luminal B, Basal-like, HER2-like, which have different prognostic and therapeutic implications [5]. However, the research of new prognostic and predictive biomarkers for the breast cancer is one of the most important scientific goals of the translational medicine. In this context, recent studies demonstrated a significant positive correlation between BMI value and the development of breast cancer [18–20]. Nevertheless, few studies have been performed about the adipose tissue's role on the carcinogenesis in breast cancer. In addition, to the best of our knowledge, no morphological data are available about the prevalence of IDFi in breast lesions.

Therefore, the main aim of this study was to evaluate the putative association between IDFi area and breast cancer histological subtypes by using digital pathology.

Digital pathology has been useful to detect the IDFi in breast lesions. Our data showed an important difference between the IDFi in malign lesions, both *in situ* and infiltrating, if compared to the benign lesion, hyperplasia and fibrous-cystic mastopathy. Moreover, no/rare IDFi was observed in the normal breast samples. From literature is known that obesity correlates with a higher risk of developing breast cancer. In particular this correlation is stronger in post menopause women who also developed an ER + cancer [8–10]. These studies showed that the estrogen expressed by the adipocytes has an important role in the tumor's growth and development [8–10]. After menopause, the adipose tissue is the main estrogen's provider for the whole body, specifically estrogens interact with the respective receptors in the ER + tumors sustaining the cancers proliferation [21–23]. Estrogens have a role in the normal development of breast's epithelial cells since they can stimulate both ductal morphogenesis and proliferation [21–23]. However, if the epithelium is exposed to high levels of estrogen, there is a higher proliferative stimulation which can lead to errors and mutations during the replication process, causing the carcinogenesis [21–23]. In agreement with this evidence, we found a positive association between the presence of IDFi and the tumor aggressiveness (histological grade). Specifically, we noted a significant increase of IDFi value in G3 infiltrating carcinomas respect to G2. On note, no difference has been observed comparing the IDFi values between *in situ* and infiltrating lesions (regardless of histological grade). This can be related to the similar histological/biological characteristics of breast *in situ* carcinomas and G1 infiltrating lesions, also in term of IDFi value. Indeed, in this study, we frequently observed very low value of IDFi in G1 infiltrating breast carcinomas.

Thus, the adipose tissue seems to correlate not only with a higher risk in developing breast cancer but also a higher risk of having relapses. As further confirmation of this, we observed that breast lesions with high IDFi were characterized by a ki67 index > 50%. Ki-67 is an important prognostic factor as well as an its nuclear antigen that is often expressed by proliferative cells [24]. There are evidences that patients showing an iper-expression of this protein have an higher risk of having a relapse [24]. According to several studies, the adipose tissue is now one of the main local estrogen's producers, that interacts with ER receptor in cancer's cells. This leads to a higher proliferation and growth rate, which is also confirmed by a high correlation between the ER and Ki67 prognostic factors. In the adipose tissue Cytochrome P450 acts as a catalyzer for estrogen's biosynthesis, its presence seems to corelate with a higher probability of developing a breast cancer ER + after menopause [25].

A very interesting data of our study concern the association between IDFi and the presence of breast microcalcifications. Indeed, we and others groups recently highlighted the possible cellular and molecular mechanisms involved in the formation of breast microcalcifications [26–31]. In particular, calcification made of hydroxyapatite seem to be produced by breast cancer cells showing an osteoblast-like phenotype, the Breast Osteoblast-Like Cells (BOLCs) [32]. In recent papers, Scimeca et al. displayed that BOLCs were ER positive cancer cells [33] whose presence increases risk of the formation of bone metastasis within 5 years from diagnosis [34]. Thus, data here reported allow us to speculate about the possible involvement of estrogen IDFi-related in both BOLCs origin and calcifications production.

5. Conclusion

In conclusion, for the first time, this study highlighted the association between IDFi and both the occurrence and progression of breast cancer. These data, if confirmed, can support the idea that the presence of IDFi represents a negative prognostic factor for breast cancer.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors have declared no conflicts of interest.

Acknowledgments

Manuel Scimeca is recipient of a fellowship from the "Fondazione Umberto Veronesi" (FUV). Rita Bonfiglio is recipient of a fellowship from "Centro Universitario Cattolico" (CUC).

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