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Comparison of nuclear grade and immunohistochemical features *in situ* and invasive components of ductal carcinoma of breast

Comparação do grau nuclear e perfil imunoistoquímico nos componentes in situ e invasivo de carcinoma mamário

Artigo Original

Keywords

Breast neoplasms/chemistry
Carcinoma, ductal, breast/chemistry
Immunoenzyme techniques
Receptors, estrogen/analysis
Receptor, erbB-2/analysis
keratin-5/analysis
keratin-6/analysis
Ki-67 antigen/analysis
Tumor makers, biological

Palavras-chave

Neoplasias da mama/química
Carcinoma ductal de mama/química
Técnicas imunoenzimáticas
Receptores estrogênicos/análise
Receptor, erbB-2/análise
Queratina-5/análise
Queratina-6/análise
Antígeno Ki-67/análise
Marcadores biológicos de tumor

Abstract

PURPOSE: To compare the prognostic and predictive features between *in situ* and invasive components of ductal breast carcinomas. **METHODS:** We selected 146 consecutive breast samples with ductal carcinoma *in situ* (DCIS) associated with adjacent invasive breast carcinoma (IBC). We evaluated nuclear grade and immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cytokeratin 5/6 (CK5/6), and epidermal growth factor receptor (EGFR) in both components, *in situ* and invasive, and the Ki-67 percentage of cells in the invasive part. The DCIS and IBC were classified in molecular surrogate types determined by the immunohistochemical profile as luminal (RE/PR-positive/ HER2-negative), triple-positive (RE/RP/HER2-positive), HER2-enriched (ER/PR-negative/HER2-positive), and triple-negative (RE/RP/HER2-negative). Discrimination between luminal A and luminal B was not performed due to statistical purposes. Correlations between the categories in the two groups were made using the Spearman correlation method. **RESULTS:** There was a significant correlation between nuclear grade ($p<0.0001$), expression of RE/RP ($p<0.0001$), overexpression of HER2 ($p<0.0001$), expression of EGFR ($p<0.0001$), and molecular profile ($p<0.0001$) between components *in situ* and IBC. CK 5/6 showed different distribution in DCIS and IBC, presenting a significant association with the triple-negative phenotype in IBC, but a negative association among DCIS. **CONCLUSIONS:** Our results suggest that classical prognostic and predictive features of IBC are already determined in the preinvasive stage of the disease. However the role of CK5/6 in invasive carcinoma may be different from the precursor lesions.

Resumo

OBJETIVO: Comparar características prognósticas e preditivas entre os componentes *in situ* e invasivo de carcinomas ductais da mama. **MÉTODOS:** Selecionamos 146 amostras mamárias consecutivas com carcinoma ductal *in situ* (CDIS) associado com carcinoma invasivo (CI) adjacente. Avaliamos grau nuclear e a expressão imunoistoquímica de receptor de estrogênio (RE), receptor de progesterona (RP), receptor do fator de crescimento epidérmico humano 2 (HER2), citoqueratina 5/6 (CK5/6) e o receptor do fator de crescimento epidérmico (EGFR) em ambos componentes, *in situ* e invasor, e a porcentagem de células marcadas pelo Ki-67 no componente invasivo. CDIS e CI foram classificados nos tipos moleculares, determinados pelo perfil imunoistoquímico, como luminal (RE/RP-positivo/HER2-negativo), triplo-positivo (RE/RP/HER2-positivo), HER2-puro (RE/RP-negativo/HER2-positivo) e triplo-negativo (RE/RP/HER2-negativo). A discriminação entre luminal A e Luminal B não foi feita por motivos estatísticos. Correlações entre as categorias dos dois grupos foram feitas pelo método de correlação de Spearman. **RESULTADOS:** Houve significante associação entre grau nuclear ($p<0,0001$), expressão de RE/RP) ($p<0,0001$), superexpressão de HER2 ($p<0,0001$), expressão de EGFR ($p<0,0001$) e perfil molecular ($p<0,0001$) entre os componentes *in situ* e invasivo. CK5/6 mostrou distribuição distinta em CDIS e CI, apresentando significante associação com o fenótipo triplo-negativo em CI, mas uma associação negativa ente os CDIS. **CONCLUSÕES:** Nossos resultados sugerem que as características prognósticas e preditivas clássicas dos CI estão já determinadas no estágio pré-invasivo da doença. Entretanto, o papel da CK5/6 no carcinoma invasivo pode ser diferente daquele das lesões precursoras.

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Received
01/11/2013

Accepted with modifications
02/01/2013

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Introduction

Ductal carcinomas *in situ* (DCIS) are immediate precursors of most breast cancer, but they are heterogeneous regarding morphology and invasiveness risk[1]. The prevalence of DCIS has been rising in the last decades, probably due to better screening programs and now accounts for approximately 20–25% of all breast cancer diagnoses[2]. The formerly accepted linear multi-step process of breast carcinogenesis, from hyperplasia, atypical hyperplasia, and carcinoma *in situ*, to invasive and metastatic carcinoma, changed to a more complex process involving a series of stochastic genetic events that lead to distinct and divergent pathways towards invasive carcinoma[3-7]. Although the progression of DCIS to invasive breast carcinoma (IBC) is believed to be an important aspect feature of tumor aggressiveness, identification of biomarkers and molecular profiles of IBC and DCIS is yet far to be fully elucidated[6,8,9]. Previous studies indicate that DCIS may be classified in a similar manner to invasive breast cancer[10-13]. The understanding of the transition between the preinvasive and invasive stages in breast carcinomas is the key to more efficient strategies for early diagnosis and treatment, as well as it expands the knowledge about the complex mechanisms of carcinogenesis. In this study our aim was to compare the prognostic and predictive pathological features between the *in situ* and invasive components of ductal breast carcinoma.

Methods

This study was approved by the Department of Pathology Scientific Committee of the Faculdade de Medicina da Universidade de São Paulo and by the Ethical Committee for Research Projects of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (CAPPesq, process 2011/14741-7). As the study was retrospective, informed patient consent was waived and any form of patient identification was abolished.

We selected breast samples from patients with confirmed diagnosis of IBC after an initial sample represented by DCIS only. Cases were obtained from the files of the Division of Surgical Pathology of Faculdade de Medicina da Universidade de São Paulo in the period from 2000 to 2009. All tissues had been fixed in 10% buffered formaldehyde and embedded in paraffin. The slides were rigorously reviewed and classified by the same pathologist with expertise in breast pathology (FNA). For cases with discordant interpretation in relation to original report, a consensus was determined by simultaneous examination under a dual-head microscope (FNA and FMC). We included only carcinomas of non-special type according criteria of the histological classification of tumors of World Health Organization, 2012[14]. Carcinomas of special types and cases with insufficient material to immunohistochemistry evaluation, signs of tissue autolysis and from pregnant patients were excluded from the study. We obtained 146 breast samples that met the criteria of inclusion. The age of the patients ranged from 29 to 87 years (median=59 years). Nuclear grades 1 (G1) and 2 (G2) were grouped as low-grade category, while nuclear grade 3 (G3) was defined as high-grade category (Figure 1). Immunohistochemistry was performed on 3 µm-thick histological sections containing *in situ* and invasive neoplasia. The source and dilutions of the antibodies and epitope retrieval methods used are listed in Table 1. Novolink® was used as the dectection system (Leica, Bannockburn, IL, USA). Nuclear positivity was considered specific for estrogen receptor (ER) (Figure 1B and 1E), progesterone receptor (PR) and Ki-67; membranous positivity for human epidermal growth factor receptor 2 (HER2) (Figure 2) and epidermal growth factor receptor (EGFR), and cytoplasmic, for cytokeratin 5/6 (CK5/6) (Figure 1). Lesions with at least 10% of cells stained were considered positive for ER and PR. For CK5/6 expression we considered any positivity above 1% of epithelial cells. For HER2 and EGFR we only considered samples positive if they scored 3+ according to American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) recommendations[15].

An approximation of breast cancer molecular subtypes was used according to the following immunohistochemical surrogate criteria modified from St. Gallen consensus[16]: Luminal A (ER/PR-positive and Ki-67<14%), Luminal B (ER/PR-positive, HER2-negative and Ki-67≥14%), triple-positive (ER/PR/HER2 positive), HER2-enriched (ER/PR-negative and HER2-positive), and triple-negative (ER/PR/HER2-negative) (TN). For DCIS we considered luminal A as being the lesions with at least 50% of ER and/or PR positive neoplastic cells, and HER2 negative; Luminal B as being the lesions with less than

Table 1. Source, dilutions of the antibodies and epitope retrieval methods used

Antigen	Clone	Dilution	Antigen retrieval	Manufacturer
ER	SP1	1:500	PC, 9 min with citric acid pH 6.0	ThermoScientific
PR	PgR636	1:1000	PC, 9 min with citric acid pH 6.0	Dako
HER2	SP3	1:100	PC, 15 min with citric acid pH 6.0	ThermoScientific
Ki-67	MIB1	1:600	PC, 8 min with citric acid pH 6.0	Dako
CK 5/6	D5/16B4	1:100	MO, 3.3 min with citric acid ph 6.0	Dako
EGFR	31G7	1:200	0.1% Pronase, 15 min	Zymed

ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; CK: cytokeratin 5/6; EGFR: epidermal growth factor receptor; PC: pressure cooker; MO: microwave oven.

50% of ER and/or RP positive cells, and HER2 negative; Triple-positive as being ER and/or PR positive associated with HER2 positivity; HER2-enriched as being ER and/or PR negative and HER2 positive, and TN as being negative for ER, PR and HER2. The subgroups luminal A and B were grouped as luminal category, as only three cases expressed less than 50% of hormonal receptors.

Associations between CK5/6 with the TN phenotype were determined by chi-square test and *Odds Ratio* with 95% confidence interval was calculated for the DCIS and IBC. Correlations between the categories of DCIS and IBC were made using the Spearman correlation method. Statistical analyses were performed using MedCalc for Windows (version 11.5.0.0; MedCalc Software, Mariakerke, Belgium), and a p-value less than 0.05 was considered significant.

Results

The distribution of the variables among *in situ* and invasive components of our cases of breast carcinoma is summarized in Table 2. Three cases of IBC G3 were associated with DCIS G2. All DCIS cases revealing G3 were associated with G3 in the invasive component as well. Two ER-negative DCIS cases showed invasive component ER-positive. Only one case of DCIS HER2-positive was associated with IBC HER2-negative. This was a 46 years-old patient with a luminal B tumor presenting with ER-negative, PR-positive and 30% cell proliferation index by Ki-67 immunostaining. Fifteen DCIS cases expressed CK5/6, but only 5/15 expressed this cytokeratin in the invasive component. On the other hand, of the 14 IBC with expression of CK5/6, 7 expressed it also in the DCIS.

The expression of CK5/6 was associated with the triple-negative phenotype in IBC (OR=7.8; 95%CI 2.4–25.3; p=0.0006). Oppositely, the expression of CK5/6 was negatively associated with TN phenotype among DCIS (OR=0.2; 95%CI 2.4–25.3; p=0.02). Only 2 cases out of 16 DCIS-EGFR-positive were negative for this marker in the IBC. Among the 100 cases of DCIS without expression of EGFR, only 5 had an invasive component positive for this growth factor. All TN DCIS were associated with TN IBC. Only one TN breast cancer had a luminal DCIS component characterized by G3 and presence of ER/PR in 10% of the tumor cells. The basal-like profile defined by ER/PR/HER2 negative and CK5/6 and/or EGFR positive was identified in 10 cases of DCIS, all of them with the same profile in the invasive component. There were 2 cases of IBC TN basal-like with an unknown status of the DCIS component, and 3 cases with a TN non-basal DCIS. Six cases of TN DCIS with non-basal-like phenotype were associated with 3 basal-like and 3 non-basal-like phenotypes in the invasive components.

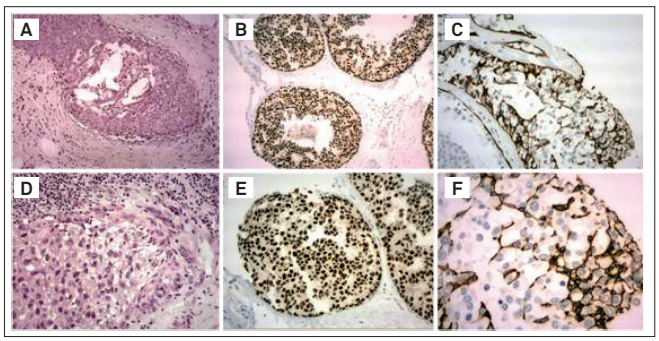

Figure 1. Upper row shows a low grade DCIS (A), ER-positive (B) and CK 5/6-positve (C). The second row presents a high grade DCIS (D), ER-positive (E) and CK5/6-positive (F).

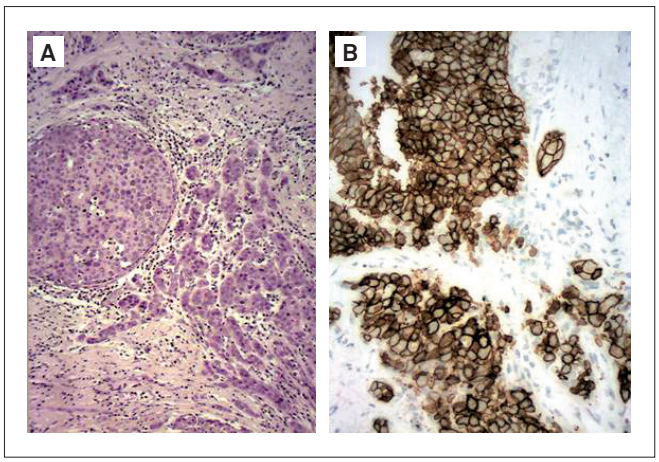

Figure 2. HER2 score 3+ in DCIS (A) and invasive carcinoma (B), both components nuclear grade 3.

Table 2. Comparison of pathological and immunohistochemical features *in situ* and invasive components of 146 ductal carcinomas

Variables		Carcinoma *in situ*	Invasive carcinoma
Nuclear grade	G1 or G2 (low grade)	95	92
	G3 (high grade)	51	54
ER/PR	Positive	104	106
	Negative	42	40
HER2	Positive (score 3)	27	26
	Negative (score 0 or 1)	119	120
CK 5/6	Positive	15	14
	Negative	118	132
	Non-available	13	0
EGFR	Positive	16	22
	Negative	100	107
	Non-available	30	17
Immunohistochemical profile	Luminal (A+B)	98	98
	Triple-positive	6	8
	HER2-enriched	21	18
	Triple-negative	21	22
Triple-negative	Basal-like profile	10	15
	Non-basal-like profile	6	7
	Non-available	5	0

ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; CK: cytokeratin 5/6; EGFR: epidermal growth factor receptor.

Table 3. Correlation between pathological and immunohistochemical features of ductal carcinoma *in situ* and adjacent invasive carcinoma (n=146)

Variables		p-value	Rho*	95%CI for Rho
Nuclear grade	Low (G1 or G2) High (3)	<0.0001	0.96	0.94–0.97
ER/PR	Positive Negative	<0.0001	0.93	0.91–0.95
HER2	Positive Negative	<0.0001	0.91	0.88–0.94
EGFR	Positive Negative	<0.0001	0.76	0.68–0.83
Molecular profile	Luminal (A+B) Triple-positive HER2 Triple-negative	<0.0001	0.96	0.95–0.97

*Spearman correlation method; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; CK: cytokeratin 5/6; EGFR: epidermal growth factor receptor.

There was a significant correlation between nuclear grade (p<0.0001), expression of ER/PR (p<0.0001), overexpression of HER2 (p<0.0001) and molecular profile (p<0.0001) between components *in situ* and invasive. The value of the correlation coefficient ranged from 0.918 (HER2) to 0.967 (molecular profile) (Table 3).

Discussion

In this study we observed similar distribution of the nuclear grade and immunohistochemical variables in DCIS and the correspondent invasive component. This observation suggests that these classical prognostic and predictive factors may be predetermined in the preinvasive stage of the disease.

The management of breast cancer is largely based on some clinical and pathological parameters, including age of patient, size of tumor, nodal status, histologic and nuclear grade, and immunohistochemical evaluation of ER, PR, HER2, and Ki-67, either as individual information, or as panel of markers to approximate the molecular subtyping classification[16]. DCIS are genuine precursors of breast cancer, but the mechanisms involved in this transition are mostly unknown. DCIS is a very heterogeneous disease with variable risk of invasion as well of recurrence, not to mention that one half of all recurrences occur as invasive cancer[17,18].

According to our results the phenotype of IBC is very similar to the *in situ* component, suggesting that the classical prognostic and predictive factors are determined previously to the invasive capacity of the precursors. Similar results have been presented in the literature[10,11,19,20]. Ottesen found similar morphology, immunohistochemistry, and DNA ploidy both in DCIS and the invasive component[19]. Park et al.[20] compared HER2 status between *in situ* and invasive component of 270 breast carcinomas and found a high concordance of 98.5 and 99.3%, respectively by FISH and immunohistochemistry. Steinman et al.[21] studied ER, PR, HER2, EGFR and several cytokeratins, including CK5/6, in 96 cases of DCIS with co-existing invasive carcinoma and they found a high rate of concordance ranging from 92.3% for ER to 100% for HER2 and EGFR. Our findings support the evidence that molecular changes implicated in the progression from *in situ* status to invasive one occur before morphological manifestation of invasiveness. Interestingly, among these classical parameters, nuclear grade is one of the most powerful prognostic and predictive factors and it is associated with distinct genetic changes[22-25]. According to some studies, it is possible that even molecular changes described in DCIS, although could be related to grade, are not determinant of invasion. Moelans et al.[26] compared copy numbers changes in 21 breast cancer related genes and the methylation status of 25 breast cancer-related genes[27] between laser-microdissected ductal carcinoma *in situ* (DCIS) and adjacent invasive ductal cancer (IDC) lesions. These authors did not observe significant differences between DCIS and adjacent IBC, suggesting that DCIS is genetically as advanced as its invasive counterpart. In an elegant study, Muggerud et al.[12] analyzed gene expressions patterns of 31 pure DCIS, 36 pure invasive cancers and 42 cases of DCIS with invasive cancer. The authors found a DCIS signature associated to gene expression characteristics more similar to advanced tumors. This set of genes was independent of grade, ER-status, and HER2-status, and it was suggestive of several processes related to the re-organization of the microenvironment[12]. In our opinion one of the most promising way to investigate the tendency of breast carcinoma to invade the stroma is to try to understand the factors related to interaction of the tumor cells with the microenvironment.

Although nuclear grade is considered the most important parameter to classify DCIS together with tumor size and margins status, it becomes evident that it is not a good predictor of invasive potential. Holmes et al.[28] studied 141 patients who underwent conservative surgery with clear margins for DCIS. They observed 60 recurrences occurred with a median follow-up of 191 months. After multivariate analysis, HER2 score 3+ was associated with reduced time to recurrence. However, no pathological or immunohistochemical characteristic was predictive of recurrence. The comprehensive systematic review conducted by Lari and Kuerer[29] to identify the

role of biological markers in DCIS, including steroid receptors, proliferation markers, cell cycle regulation and apoptotic markers, angiogenesis-related proteins, epidermal growth factor receptor family receptors, extracellular matrix-related proteins, and COX-2, failed to find any useful marker.

After the molecular classification of breast into intrinsic subtypes luminal A, Luminal B, HER2 and basal-like[30] and the demonstration of immunohistochemical surrogates[31], several groups dedicated to investigate the role of the same profiles in DCIS[10,13,32]. Our results show a similar distribution of the molecular subtypes between *in situ* and invasive carcinomas. The more controversial subtype is the basal-like. According Nielsen et al.[31], the better determination of the basal-like subgroup is based on TN phenotype associated to expression of CK5/6 and/or EGFR. We studied a substantial number of TN DCIS (21 cases), all of them were associated with TN in the invasive component. However, our results indicate that the significance of basal cytokeratin 5/6 in DCIS may be different of that in invasive carcinomas. In fact, CK5/6 was negatively associated with the TN phenotype and more prevalent in the other subtypes. Further studies are needed to clarify the role of this cytokeratin in DCIS.

Acknowledgement

This study was supported by grants from FAPESP (São Paulo Research Founding), process number 2011/14741-7.

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