Male and Female Breast Cancer – Differences in DNA Ploidy, p21 and p53 Expression Reinforce the Possibility of Distinct Pathways of Oncogenesis

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Key Words

Male/female breast cancer • p21 • p53 • pRb • Ki-67 • Ploidy

Abstract

Aim: The purpose of this study was to compare the immunohistochemical profile of cell cycle inhibitors of G1/S phase transition (p21, p53 and pRb), Ki-67 proliferation marker and DNA ploidy in male (MBC) and female breast cancer (FBC).

Material and Methods: One hundred patients (50 non-consecutive cases of FBC and an equal number of MBC) were selected according to homogeneous features regarding age, histological type, tumour grading, nodal status and absence of neoadjuvant therapy. The expression of p21, p53, pRb and Ki-67 was assessed by immunohistochemistry, and DNA ploidy was analysed by flow cytometry. Correlations between variables were evaluated using the χ^2 test. **Results:** The incidence of DNA aneuploid, p21-positive and p53-negative tumours was significantly higher in MBC than in FBC; pRb and Ki-67 revealed no statistically significant differences between the two entities. In MBC, high tumour grade correlated with aneuploidy, Ki-67 and pRb positivity; ploidy and p53 were also associated. In FBC, only ploidy and grade showed a strong correlation. **Conclusion:** The significant dissimilarities regarding DNA ploidy, p21 and p53 in these quite homogeneous groups of FBC and MBC point to differ-

ent genomic instability and to differences in cell cycle proliferative control, reinforcing the view of somewhat distinct tumour oncogenesis.

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Introduction

The molecular mechanisms underlying breast cancer pathogenesis and their clinical correlation are mostly unknown, both in male and female patients [1–3]. Interactions between cell cycle inhibitors and tumour cell proliferation markers have been reported as critical to clarify relevant aspects of cancer biopathology, but their true role in oncogenesis and tumour progression, as well as in the clinical effects of chemotherapeutic agents, is still unclear [4–6].

The most well-studied gene is p53, which has been implicated in DNA repair and apoptosis in response to DNA damage, as well as in cell cycle regulation, differentiation and angiogenesis; however, many questions about p53 response pathways continue to be unsolved [7]. Its transcriptional regulated p21 protein, besides being related to cell repair and apoptosis, appears to promote either growth arrest or proliferation in dependence of the cellular context and mediate the activation of the estrogen-

Table 1. Comparison between FBC and MBC – similar clinicopathological characteristics of two groups of 50 patients with invasive ductal carcinoma

Characteristics	Female		Male		p value
	n	%	n	%	
Age					0.074
≤50 years	13	26	6	12	
>50 years	37	74	44	88	
Tumour grading					0.829
G1	16	32	15	30	
G2/G3	34	68	35	70	
Nodal status					0.622
Negative	24	48	18	43	
Positive	26	52	24	57	

Note that in 8 MBC cases we had no information about nodal status.

signalling pathway in estrogen receptor-negative neoplastic cells [4, 6, 8–10]. The product of the retinoblastoma gene (pRb) is also involved in the cell cycle control, and its expression seems to parallel proliferative activity in breast carcinomas [11]. Furthermore, it has widely known biological and clinical implications of high cell proliferation and DNA aneuploidy in breast cancer, since both were found to be associated with aggressive clinical behaviour [12, 13].

The purpose of this study was to compare the immunohistochemical profile of cell cycle inhibitors of G1/S phase transition (p21, p53 and pRb), Ki-67 cell proliferation marker and DNA ploidy between two groups of patients with invasive ductal female (FBC) and male breast cancer (MBC), selected according to relatively homogeneous clinicopathological and therapeutic criteria. All the parameters studied were also correlated within the groups of male and female cancer.

Patients and Methods

Clinicopathological Data

The retrospective series of 100 patients investigated included two groups (50 MBC and an equal number of FBC cases) retrieved from the files of the Instituto Português de Oncologia, Lisboa, Portugal. To improve the reliability of the comparative analysis between the two groups, the patients were selected according to homogeneous criteria, namely, the absence of neoadjuvant therapy, the same histological type (invasive ductal carcinomas of no special type – NOS) [14], similar nodal status ($p = 0.622$) evalu-

ated according to the pTNM classification [15], and similar tumour grading ($p = 0.829$), estimated using the Elston and Ellis system [16] (table 1). In MBC, we had no information about nodal involvement in 8 cases, and 17 patients showed cutaneous ulceration at diagnosis (stage III). The age of the patients (mean age in the FBC group was 57.8 years, range 40–81, and in the MBC group, 63.6 years, range 39–87), although maintaining the usual frequency of MBC in an older age group compared with FBC, was not statistically different ($p = 0.074$), reinforcing the homogeneous features of the two study groups (table 1).

The information about therapeutics and age of the patients was obtained from the patients' medical records.

Immunohistochemistry Assays

Immunocytochemical analyses were performed on formalin-fixed paraffin-embedded material using the streptavidin-biotin complex peroxidase technique [17]. Pretreatment procedures included endogenous peroxidase blocking with 3% hydrogen peroxide in methanol and antigen retrieval using either a pressure cooker (1 min) and 0.01 M sodium citrate buffer, pH 6 (for p53 and Ki-67), or a 750-watt microwave oven (20 min) and a low pH solution (S1699 target retrieval solution, Dako, Glostrup, Denmark) (for p21 and pRb). Primary monoclonal antibodies used comprised anti-p53 clone D07 (Novocastra Laboratories, Newcastle upon Tyne, UK; 30 min, 1:50 dilution, at room temperature), anti-p21 clone SX118 (Dako; 60 min, 1:25 dilution, at room temperature), anti-pRb clone Rb1 (Dako; overnight, 1:500 dilution, at 4°C) and anti-Ki-67/7B11 clone (Zymed Laboratories, San Francisco, Calif., USA; 30 min, 1:50 dilution, at room temperature). The StreptABComplex/HRPDuet Mouse/Rabbit system (Dako K0492), which includes secondary biotinylated antibody and streptavidin-biotinylated horseradish peroxidase, was used as amplifier reagent. The chromogen solution (DAB-hydrogen peroxide; Dako K5001) was applied for staining visualization (brown colour), and the counterstaining was performed with Mayer's haematoxylin (blue colour).

Specific staining of nuclear proteins was evaluated by 2 investigators, semiquantitatively, using a 2-headed microscope. The immunoreactivity of the sample was analysed by counting at least 500 tumour cells. To improve interlaboratory comparison of data, we adopted 10% as the cut-off point for the staining classification of p21, p53, pRb ($\leq 10\%$ /negative vs. $>10\%$ /positive) and Ki-67 ($\leq 10\%$ /low vs. $>10\%$ /high proliferative tumours).

DNA Flow Cytometry

In FBC, flow cytometric analysis was performed on representative frozen samples of tumour tissue obtained at surgical resection, according to a previously reported procedure [12]. In MBC, DNA flow cytometry was performed on paraffin-embedded material, according to the method of Hedley et al. [18], with slight modifications. Following the review of haematoxylin and eosin-stained slides, the blocks predominantly composed of tumour were selected, and 50- μm -thick representative sections were cut, deparaffinised in xylene and rehydrated in decreasing concentrations of ethanol. Disaggregation was performed enzymatically by treating sections with 0.5% pepsin pH 1.5 in a water bath at 37°C with frequent vortexing. The samples were washed in distilled water and filtered with a 55- μm nylon mesh. The following steps included cell counting, nuclei staining with 50 $\mu\text{g}/\text{ml}$ propidium iodide (Sigma), treated with 1 mg/ml ribonuclease in phosphate-

buffered saline, 0.05% NP40 non-ionic detergent (Sigma) (overnight at 4°C, in the dark), filtration, and finally, flow cytometer analysis. Nuclear DNA ploidy was evaluated on an Epics Profile II flow cytometer (Coulter Electronics, Hialeah, Fla., USA). Fluorescent microbeads were used for daily calibration of the instrument (Flow-Check Fluorospheres, Beckman-Coulter). DNA histograms were analysed using the Multicycle program (Phoenix Flow Systems, San Diego, Calif., USA), which includes a multiple option cell cycle fitting that automatically determines the DNA index. The tumours were classified dichotomically (diploid vs. aneuploid) according to the DNA ploidy pattern. On histograms obtained from paraffin-embedded tissue, the first peak observed was assumed as the G0/G1 diploid population. Histograms with a coefficient of variation >6% and/or a high percentage (>20%) of aggregates and debris were excluded from the study.

Statistical Analysis

Correlations between variables were assessed using the χ^2 test and Fisher's exact test when appropriate. In order to evaluate homogeneous groups of FBC and MBC, a comparative analysis of patients' age (≤ 50 vs. >50 years), tumour grading (G1 vs. G2/G3) and nodal status (positive vs. negative) was previously performed in both groups to confirm the eligibility of patients.

The frequency tables were organised for the following parameters: p53, p21, pRb (positive vs. negative), Ki-67 (low vs. high cell proliferation) and DNA ploidy (aneuploidy vs. diploidy). p values <5% were considered as the significance level.

Results

Table 2 illustrates the comparative study between the two groups of FBC and MBC patients in relation with immunohistochemical features and DNA ploidy. The MBC group showed a higher frequency of DNA aneuploid tumours, with statistical significance ($p < 0.001$). The incidence of p21-positive (48/50) and p53-negative (48/50) neoplasms in MBC was also significantly higher than in the FBC group ($p < 0.001$ and $p = 0.014$, respectively). pRb and Ki-67 immunorexpression were quite similar between MBC and FBC.

Table 3 summarizes the associations between parameters within both groups. In FBC, high-grade tumours strongly correlated with DNA aneuploidy ($p < 0.001$) and showed a trend of being associated with high Ki-67 cell proliferation, which did not reach statistical significance ($p = 0.071$). In MBC, the two p53-positive tumours showed a DNA diploid pattern ($p = 0.037$). Moreover, the G2/G3 grading tumours significantly correlated with DNA aneuploidy (88.6% of cases; $p = 0.020$), pRb-positive tumours (77.8% of cases; $p = 0.035$), and Ki-67-positive/high proliferative tumours (74.3% of cases; $p = 0.020$).

Table 2. Comparison between FBC and MBC – characterization of the patient cohort by immunohistochemical features and DNA ploidy

Characteristics	Female		Male		p value
	n	%	n	%	
p53					0.014
Positive	9	18	2	4	
Negative	41	82	48	96	
p21					<0.001
Positive	29	58	48	96	
Negative	21	42	2	4	
pRb					0.159
Positive	13	26	15	30	
Negative	37	74	35	70	
Ki67					0.157
$\leq 10\%$	25	50	18	36	
$>10\%$	25	50	32	64	
DNA ploidy					<0.001
Diploid	27	54	10	20	
Aneuploid	23	46	40	80	

Table 3. Correlation between variables within FBC (italics) and MBC groups

Variables	Grade	Ki-67	Ploidy	p53	p21	pRb
Grade	–	<i>0.071</i>	<0.001	<i>0.373</i>	<i>0.867</i>	<i>0.571</i>
Ki-67	0.020	–	<i>0.128</i>	<i>0.145</i>	<i>0.085</i>	<i>0.500</i>
Ploidy	0.020	0.463	–	<i>0.261</i>	<i>0.145</i>	<i>0.380</i>
p53	<i>0.538</i>	<i>0.595</i>	0.037	–	<i>0.176</i>	<i>0.258</i>
p21	<i>0.538</i>	<i>0.595</i>	<i>0.637</i>	<i>0.921</i>	–	<i>0.268</i>
pRb	0.035	0.074	<i>0.263</i>	<i>0.309</i>	<i>0.691</i>	–

The χ^2 test and Fisher's exact test were used. Significant associations are given in bold.

Discussion

It is well established that genetic abnormalities related to proteins that control the cell cycle, together with hormones and other growth regulators, by promoting or inhibiting cellular proliferation and apoptosis, are implicated in the tumour development and progression of both FBC and MBC [7–11, 19]. Since MBC is an uncommon entity, the biological information on its oncogenesis mechanisms has been largely extrapolated from its female counterpart. The question is: do cancer growth regulators act in a similar way in cases of FBC and MBC?

Previous studies reported that MBC and FBC have some biological characteristics in common, but also exhibit significant differences [1, 3, 20, 21]. It still remains controversial whether the development of MBC parallels that of FBC, and how genetic alterations affect the molecular pathogenesis and patients' management.

In our study, we selected two relatively homogeneous groups of male and female patients who had not received primary neoadjuvant therapy and did not show significant differences in pathological features, such as histological type, tumour grading and nodal status. The age of patients, while corroborating the documented occurrence of MBC in older patients compared with FBC [21], revealed no statistically significant difference either. The methodological approach, aiming to improve the clinico-pathological and therapeutic similarity of the two groups, allowed a more reliable comparison between different features.

The main findings of the present study related with the significantly higher incidence of DNA aneuploidy, p53-negative and p21-positive tumours in MBC.

As previously reported in another study of our group [12], DNA ploidy is strongly correlated with histological grade in FBC, which also occurred in MBC. The high rates of aneuploidy in MBC, in general associated with a poor clinical outcome [13], indicate increased genomic instability, suggesting the presence of specific chromosomal abnormalities. The differences in the DNA ploidy status between FBC and MBC were statistically significant and could reflect distinct underlying mechanisms of genetic evolution.

The reported incidence and biological impact of p53 expression are variable both in FBC and MBC [1, 2, 5, 6, 13, 22–26]. In the present study, p53 positivity was low in FBC (18%) and rare in MBC (2 cases, 4%). Despite the small number of cases, the difference is statistically significant. However, the association between p53 positivity and DNA diploidy in MBC seems surprising, possibly due to the fact that only two tumours were positive, since p53 immunoexpression is usually associated with aneuploid phenotypes and a worse prognosis [13, 27].

In our study, the percentage of abnormal p21 expression (immunohistochemical negative tumours) was significantly lower in MBC than in FBC (4 and 42% of cases, respectively), which is in keeping with previous results reported by Curigliano et al. [3]. The significant difference of p21 expression in FBC and MBC appears to be an especially important finding, as it may be an indicator of a distinct pattern of tumorigenesis in these groups of tumours. The coexistence of high p21 positivity (96% of the

cases) and the lack of p53 overexpression (96% of the cases), reflecting normal biological mechanisms, strongly suggest that alterations to the p53 signalling pathway have very little influence on MBC tumour development.

In the literature, variable expression of p21 protein and its diverse correlation with other parameters and prognosis were reported in breast cancer, which could be explained by the different methods of immunohistochemical detection and interpretation criteria used [3, 5, 6, 9, 24, 27–30]. We adopted the most reported cut-off point for staining interpretation, to make the interlaboratory comparison of results easier. In this study, as in others [6, 29, 30], p21 expression was not correlated with other cell cycle regulatory proteins, Ki-67 proliferation marker and DNA ploidy, keeping its biological role in cancer progression unclear.

Our data revealed no statistically significant differences between the two groups in relation to pRb expression and Ki-67 tumour proliferative activity. We found a high percentage of pRb-negative cases, which was similar in FBC and MBC (74 and 70%, respectively), suggesting that most tumours presented in their pRb-inactive phosphorylated form. There was a significant correlation in MBC between pRb and grade, but we have failed to previously demonstrate reported associations of pRb with Ki-67 and p53 [11, 23]. The cell proliferation, as assessed by Ki-67, correlated with tumour grade in MBC and showed a trend in FBC. However, in accordance with data of Pich et al. [13], it was quite similar in the two groups of breast cancers in our series.

In conclusion, our results showed that the cell cycle regulatory proteins investigated in FBC and MBC have very few similarities, suggesting the existence of independent mechanisms of tumorigenesis. In addition, the significant dissimilarities regarding DNA ploidy, p21 and p53 expression in these quite homogeneous groups of FBC and MBC point to different genomic instability and to differences in the cell cycle proliferative control, reinforcing the view of somewhat distinct pathways of tumour oncogenesis.

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