Breast Cancer Research and Treatment (2005) 92: 77–80
DOI 10.1007/s10549-005-2114-9

Report

The S-phase fraction of the aneuploid cell subpopulation is the biologically relevant one in aneuploid breast cancers

F. Martinez-Arribas[1], M.J. Nuñez-Villar[1], A.R. Lucas[1], J. Sanchez[1,2], A. Tejerina[1], and J. Schneider[1,3]
[1]*Fundación Tejerina, Centro de Patologia de la Mama, Madrid, Spain;* [2]*Universidad de Alcala, Madrid, Spain;* [3]*Universidad Rey Juan Carlos, Madrid, Spain*

Key words: breast, cancer, ploidy, S-phase

Summary

Background. In the case of DNA-aneuploid tumors there are no clear guidelines as to which S-phase fraction is the more relevant one: that corresponding to either the diploid or the aneuploid population, or rather an average of both.

Materials and methods. We studied 280 breast cancer specimens from previously untreated patients. Histologically, 231 were ductal infiltrating carcinomas, 30 lobular infiltrating carcinomas and 19 corresponded to other, less frequent varieties. Postsurgically, 164 cases (58.6%) were classified as T1, 87 (31.1%) as T2 and 7 as T3. The remaining 22 cases were multifocal, diffuse tumors. Flow cytometry was performed on fresh tumor tissue, and immunohistochemistry for hormone receptors, Ki67, c-erb-B2 and p53 on paraffin-embedded material.

Results. In diploid tumors, a high S-phase (above the 75th percentile) correlated significantly with Ki67 expression ≥20% ($p < 0.0001$). In aneuploid tumors, however, this was only the case for the aneuploid fraction of tumor cells ($p < 0.0001$). A high S-phase of diploid tumors correlated directly and significantly with a high histologic grade ($p = 0.04$), a high nuclear grade ($p = 0.01$), tumor size ($p = 0.0008$), and inversely with estrogen ($p < 0.0001$) and progesterone ($p < 0.0001$) receptor expression. In aneuploid tumors, the aneuploid tumor fraction showed a direct and significant correlation with a high histologic grade ($p = 0.005$), a high nuclear grade ($p = 0.001$), mutant p53 expression ($p = 0.0009$), and inversely with estrogen ($p < 0.0001$) and progesterone ($p = 0.0001$) receptor expression. A high S-phase of the diploid cell fraction of aneuploid tumors, on the other hand, just showed an inverse correlation with high nuclear grade of the tumors ($p = 0.02$), and none whatsoever with all other tested parameters.

Introduction

Cellular proliferation is an important prognostic parameter in breast cancer [1–6]. In fact, some authors believe that it is one of the most relevant independent predictors of survival in this kind of tumor [7]. Measurement of the dividing tumor cell fraction in S-phase by means of flow cytometry is an established method for assessing the proliferative activity of breast cancer, and has been shown by us in the past to be an independent prognostic factor for DNA-diploid tumors [8]. In the case of DNA-aneuploid tumors, however, the situation is still controversial. In fact, there are no clear guidelines as to which S-phase is the more relevant one, when both types of cell populations are present: that corresponding to either the diploid or the aneuploid population, or rather an average of both. In an attempt to clarify this, we have measured proliferation in a series of breast cancers both by means of flow cytometry and immunohistochemistry. The results have then been correlated with all available clinical and biological parameters of the tumors. Since a very large proportion of the latter were early (pT1) carcinomas, with an excellent general prognosis, we could not use survival as prognostic endpoint, due to insufficient follow-up. Instead, we used axillary node invasion and tumor size as second-best surrogates of final outcome of the patients.

Materials and methods

We studied 280 breast cancer specimens from previously untreated patients operated upon at Fundación Tejerina, Madrid, Spain, between January 2000 and December 2003. All patients gave written informed consent for the research use of the aforementioned samples. Histologically, the vast majority corresponded to ductal infiltrating carcinomas (231), followed by lobular infiltrating carcinomas (30) and other, less frequent varieties (19). As has been mentioned earlier, a large proportion (164 cases, 58.6%) were classified postsurgically as T1, 87 (31.1%) as T2 and 7 as T3. The remaining 22 cases

corresponded to multifocal, diffuse tumors. Metastatic axillary nodes were present in 99 instances.

Flow cytometry was invariably carried out on fresh tumor tissue, whereas for the immunohistochemical detection of hormone receptors, Ki67, c-erb-B2 and p53 we used paraffin-embedded material.

Immunohistochemistry

The immunohistochemical technique employed was the standard one at our laboratory, and has also been described elsewhere [9]. Briefly, 5-μm paraffin sections were mounted on poly-L-lysine coated slides for heat-induced epitope retrieval ('HIER' technique) in citrate buffer. We used the same, commercially available streptavidin–biotin-peroxidase kit (Histostain-SP, Zymed, San Francisco, CA, U.S.A.) throughout the whole procedure, to ensure uniformity of the results. The antibodies employed were as follows: NCL-CB11 (c-erb-B2), NCL-ER-6F11 (estrogen receptor), NCL-p53-D07 (p53), all from Novocastra Laboratories, Newcastle, UK; prediluted MIB1 (Ki67) and PR-2C5 (progesterone receptor) from Zymed, San Francisco, CA, USA. The incubation time was 1 h at room temperature in a humid chamber for all antibodies, which, apart from the prediluted MIB1-Ki67 solution, which was directly used as supplied, were employed at following dilutions: NCL-CB11 (c-erb-B2): 1:40; NCL-ER-6F11 (ER): 1:100; NCL-p53-D07: 1:100. The evaluation of nuclear staining patterns (ER, PR, Ki67 and p53) was straightforward, since specimens positive for ER, PR or p53 always showed specific staining in more than 20% of tumor cells. The Ki67 labeling index was expressed as the percentage of reactive tumor cells. The tumors were considered c-erb-B2-positive when more than 10% of cells showed specific membrane staining.

Flow-cytometry

The procedure was carried out always on fresh tumor tissue, which was kept in phosphate-buffered saline (PBS) at 4 °C for less than 24 h. After having been obtained, according to protocols previously described by us [10]. It was first finely minced with a scalpel blade, mixed with 2 ml of DNA-prep Stain reactant and 100 μl DNA-prep LPR reactant (both from Coulter Corporation, Miami, FL, USA), and incubated for 30 min at 37 °C. The resulting mixture was then filtered through a 50-μm pore filter and was ready for cytometric analysis in a Coulter EPICS XL cytometer (Coulter Corporation, Miami, FL, USA).

The analysis of the obtained histograms was carried out with the help of the MultiCycle DNA Cell Cycle Analysis software package (Phoenix Flow Systems, San Diego, CA, USA). Tumors were considered diploid when the DNA-index obtained was 1.0, and aneuploid for any diverging value, including tetraploid tumors, with a DNA-index of 2.0.

Statistics

For comparative purposes with qualitative variables, the S-phase value was divided into 'high' and 'low' values, using the 75th percentile of either the diploid or aneuploid fraction as cutoff. The statistical analysis was performed using the GraphPad Prism biomedical statistical package (GraphPad Software, Inc., San Diego, CA, USA). Values were considered significant, when p was < 0.05.

Results

Out of the 280 analyzed tumors, the DNA-index could be successfully evaluated in 275 (151 (54.9%) – diploid; 124 (45.1%) – aneuploid) and the S-phase fraction in 273. The Ki67 labeling index was evaluated in 277 cases (Table 1). In aneuploid tumors we measured the S-phase fraction corresponding to both the diploid and the aneuploid cell population, calculating the corresponding 75th percentile. The 75th percentile of the S-phase fraction was 7.2% for diploid tumors, whereas for aneuploid tumors, it was 16.4% for the diploid population and 12.6% for the aneuploid cell cohort.

Table 1. Correlations between the different S-phases of all cell subpopulations and the clinical and biological features of the tumors

	Diploid tumors		Aneuploid tumors			
	Diploid S-phase		Diploid S-phase		Aneuploid S-phase	
	r	*p*	*r*	*p*	*r*	*p*
Histologic grade	0.18	0.04	−0.007	0.93	0.26	0.005
Nuclear grade	0.21	0.01	−0.2	0.02	0.3	0.001
Axillary metastasis	0.02	0.79	−0.08	0.32	−0.08	0.33
ER	−0.37	< 0.0001	0.06	0.46	−0.48	< 0.0001
PR	−0.35	< 0.0001	0.11	0.22	−0.37	0.0001
c-erb-B2	0.11	0.18	0.03	0.67	0.17	0.053
p53	0.14	0.09	0.04	0.66	0.3	0.0009
Tumor size	0.28	0.0008	−0.002	0.98	0.1	0.29

DNA-aneuploidy of the tumors correlated directly and significantly with a high histologic grade ($p = 0.01$), a high nuclear grade ($p < 0.0001$), axillary node invasion ($p = 0.007$), c-erb-B2 expression ($p = 0.008$), mutant p53 expression ($p = 0.007$), tumor size ($p = 0.01$), and inversely with estrogen ($p = 0.006$) and progesterone ($p = 0.002$) receptor expression.

In diploid tumors, a high S-phase (above the 75th percentile) correlated significantly with Ki67 expression ≥20% ($p < 0.0001$). In aneuploid tumors, however, this was only the case for the aneuploid fraction of tumor cells ($p < 0.0001$).

Moreover, a high S-phase of diploid tumors correlated directly and significantly with a high histologic grade ($p = 0.04$), a high nuclear grade ($p = 0.01$), tumor size ($p = 0.0008$), and inversely with estrogen ($p < 0.0001$) and progesterone ($p < 0.0001$) receptor expression.

In aneuploid tumors, the aneuploid tumor fraction showed a direct and significant correlation with a high histologic grade ($p = 0.005$), a high nuclear grade ($p = 0.001$), mutant p53 expression ($p = 0.0009$), and inversely with estrogen ($p < 0.0001$) and progesterone ($p = 0.0001$) receptor expression. A high S-phase of the diploid cell fraction of aneuploid tumors, on the other hand, just showed an inverse correlation with high nuclear grade of the tumors ($p = 0.02$), and none whatsoever with all other tested parameters.

All results are summarized in Table 1.

Discussion

It is generally assumed that both S-phase and Ki67 expression measure the same parameter in tumors, namely proliferation. Although this is true in a general sense, they are not exactly superimposable, as we have already shown in the past [10]. Vielh et al. [7] also found that a high percentage of tumor cells expressing the Ki67 antigen was significantly associated with axillary metastasis, whereas a high S-phase was not, but was inversely associated with estrogen receptor expression, instead. We also found in the present study the same inverse correlation between a high S-phase and hormone receptor expression, but not the one described by Vielh et al. with axillary node invasion. The only cell-cycle parameter of our study associated with axillary involvement was aneuploidy of the tumors. Gilchrist et al. [11] also observed a tight relationship between aneuploidy and axillary node metastasis, as well as a significant inverse correlation with estrogen receptors, as we did. Similar results were also reported by Pinto et al. [12] for locally advanced breast cancers.

The most striking result of our study is the finding that the relatively small subpopulation of aneuploid cells present in aneuploid tumors seems to be responsible for their ominous biological features. The diploid cell fraction, conversely, usually representing the majority of cells in an aneuploid tumor, seems not to contribute significantly to its biological aggressiveness, irrespectively of its proliferative capacity, represented by its S-phase. This is at first sight surprising, considering the relevance of S-phase in purely diploid tumors, as shown by previous studies, where this parameter is a powerful independent prognostic marker [8]. However, our finding is fully in agreement with the recommendations by Kallioniemi et al. [13] who advise to study both cell subpopulations in aneuploid tumors whenever the quality of the histograms allows to do so, and to use only the S-phase corresponding to the aneuploid one for prognostic purposes. Our results also indirectly corroborate those of Chassevent et al. [2] who only studied the S-phase of the aneuploid subpopulation in tumors with a DNA-index different from 1, and found this to be associated with large tumor size, high histologic grade, axillary node invasion and hormone receptor negativity.

References

1. Camplejohn RS, Ash CM, Gillett CE, Raikundalia B, Barnes DM, Gregory WM, Richards MA, Millis RR: The prognostic significance of DNA flow cytometry in breast cancer: results from 881 patients treated in a single centre. Br J Cancer 71: 140–145, 1995
2. Chassevent A, Jourdan ML, Romain S, Descotes F, Colonna M, Martin PM, Bolla M, Spyratos F: S-phase fraction and DNA ploidy in 633 T1T2 breast cancers: a standardized flow cytometric study. Clin Cancer Res 7: 909–917, 2001
3. Hedley DW, Clark GM, Cornelisse CJ, Killander D, Kute T, Merkel D: DNA Cytometry Consensus Conference. Consensus review of the clinical utility of DNA cytometry in carcinoma of the breast. Breast Cancer Res Treat 28: 55–59, 1993
4. Kute TE, Quadri Y, Muss H, Zbieranski N, Cirrincione C, Berry DA, Barcos M, Thor AP, Liu E, Koerner F: Flow cytometry in node-positive breast cancer: cancer and leukemia group B protocol 8869. Cytometry 22: 297–306, 1995
5. Stal O, Dufmats M, Hatschek T, Carstensen J, Klintenberg C, Rutqvist LE, Skoog L, Sullivan S, Wingren S, Nordenskjold B: S-phase fraction is a prognostic factor in stage I breast carcinoma. J Clin Oncol 11: 1717–1722, 1993
6. Wenger CR, Beardslee S, Owens MA, Pounds G, Oldaker T, Vendely P, Pandian MR, Harrington D, Clark GM, McGuire WL: DNA ploidy, S-phase, and steroid receptors in more than 127,000 breast cancer patients. Breast Cancer Res Treat 28: 9–20, 1993
7. Vielh P, Chevillard S, Mosseri V, Donatini B, Magdelenat H: Ki67 index and S-phase fraction in human breast carcinomas. Comparison and correlations with prognostic factors. Am J Clin Pathol 94: 681–686, 1990
8. Romero H, Schneider J, Burgos J, Bilbao J, Rodriguez-Escudero FJ: S-phase fraction identifies high-risk subgroups among DNA-diploid breast cancers. Breast Cancer Res Treat 38: 265–275, 1996
9. Schneider J, Pollan M, Ruibal A, Jimenez E, Lucas AR, Nunez MI, Sanchez N, Tejerina A: Histologic grade and CD44 are independent predictors of axillary lymph node invasion in early (T1) breast cancer. Tumour Biol 20: 319–330, 1999
10. Martinez-Arribas F, Nunez MJ, Piqueras V, Lucas AR, Sanchez J, Tejerina A, Schneider J: Flow cytometry vs. Ki67 labelling index in breast cancer: a prospective evaluation of 181 cases. Anticancer Res 22: 295–298, 2002

11. Gilchrist KW, Gray R, Driel-Kulker AM, Mesker WE, Ploem-Zaaijer JJ, Ploem JS, Taylor SG, Tormey DC: High DNA content and prognosis in lymph node positive breast cancer. A case–control study by the University of Leiden and ECOG. (Eastern Cooperative Oncology Group).. Breast Cancer Res Treat 28: 1–8, 1993
12. Pinto AE, Andre S, Mendonca E, Silva G, Soares J: Overall survival in advanced breast cancer: relevance of progesterone receptor expression and DNA ploidy in fine-needle aspirates of 392 patients. Int J Biol Markers 18(1): 7–12, 2003
13. Kallioniemi OP, Visakorpi T, Holli K, Isola JJ, Rabinovitch PS: Automated peak detection and cell cycle analysis of flow cytometric DNA histograms. Cytometry 16: 250–255, 1994

Address for offprints and correspondence: Dr J. Schneider, Fundación Tejerina, Calle José Abascal, 40, 28003 Madrid, Spain; *Tel.:* +34-91-399-4280; *Fax:* +34-91-446-0253; *E-mail:* schneider@teleline.es