*Eur J Cancer Clin Oncol*, Vol. 23, No. 3, pp. 277–282, 1987.
Printed in Great Britain

0277–5379/87$3.00+0.00


# Aneuploid DNA Content and High S-Phase Fraction of Tumour Cells are Related to Poor Prognosis in Patients with Primary Breast Cancer

OLLI-PEKKA KALLIONIEMI,* TENHO HIETANEN,† JORMA MATTILA,‡ MATTI LEHTINEN,§ KALEVI LAUSLAHTI‡ and TIMO KOIVULA*

*Departments of Clinical Chemistry,* Radiotherapy† and Pathology,‡ University Central Hospital, Department of Biomedical Sciences,§ University of Tampere, Tampere, Finland*

**Abstract**—*The prognostic impact of DNA content and S-phase fraction (SPF) of tumour cells was studied in 93 patients with primary breast cancer. Aneuploid DNA content and high SPF were clearly associated with poor differentiation state of tumours and absence of steroid, especially progesterone receptors. Aneuploidy and high SPF tended to become more common with increasing primary tumour size, with more extensive nodal involvement and with more advanced stage of the cancer. Patients with diploid tumours had a slightly longer disease-free interval and survival than those with aneuploid tumours, whereas below median SPF as compared to above median SPF was associated with significantly longer* ($P < 0.01$) *relapse-free interval and survival in patients with stage II–III cancer. We conclude that the DNA analysis of tumour cells is a promising method for the estimation of prognosis in breast cancer patients.*

## INTRODUCTION

STATIC microspectrophotometric studies of DNA content in breast cancer cells have suggested that aberrations from the diploid DNA content are associated with poor prognosis of the patients [1, 2]. The proliferative activity of breast cancer cells as measured by their thymidine-labelling index in autoradiography also correlates with patients' prognosis [3, 4]. Recently, rapid and sensitive flow cytometric techniques have been described for the determination of both the ploidy and the S-phase fraction (SPF) of tumour cells [5, 6, 7]. Aneuploid cellular DNA content and high SPF have been implicated as poor prognostic signs in a variety of human malignancies [5, 6]. It has been suggested that the rate of relapse of breast cancer is also greater in aneuploid as compared to diploid tumours [8–11]. However, there is no information on the survival of patients with either diploid or aneuploid tumours. Furthermore, there are no reports correlating flow cytometric SPF with the rate of relapse or survival of breast cancer patients.

We have studied the prognostic value of the DNA content and SPF of tumour cells in 93 patients with primary breast cancer. The correlation of flow cytometric parameters with the stage and grade of the tumour and the estrogen and progesterone receptor status was also evaluated.

Accepted 1 August 1986.
Address for correspondence: Olli-Pekka Kallioniemi, M.D., Dept. Clinical Chemistry, University Central Hospital, SF-33520 Tampere, Finland.

## MATERIALS AND METHODS

*Patient material*

Ninety-three patients operated between 1976 and 1978 for primary breast cancer in the University Central Hospital of Tampere were included in the study. The cancer was diagnosed histologically and graded according to the WHO classification [12]. Seventy-nine of the patients had invasive ductal, 6 intraductal, 3 papillary, 2 mucinous, 1 medullary, 1 lobular and 1 adenoid cystic carcinoma. The clinical stage was classified according to the UICC classification [13]. Twenty patients had stage I, 53 stage II, 19 stage III and 1 stage IV carcinoma. The primary operation was simple mastectomy in 39 cases, modified radical mastectomy in 53 cases and tumorectomy in 1 case. Postoperative radiotherapy was given to 39 patients with stage II or greater. The patients were followed at 3-month intervals for 1 year, in 6-month intervals up to 5 years and thereafter once a year. Metastases were verified by fine-needle aspiration or surgical biopsy, chest and bone radiography and/or liver and brain scans.

After metastases patients with steroid receptor-positive tumours received hormonal therapy in the form of antiestrogens, progestins or oophorectomy. Receptor negative patients and those failing to respond to hormonal therapy received either of the two cytostate combinations: A, cyclophosphamide-5-fluorouracil-methotrexate-vincristine or B, 5-fluorouracil-doxorubicin-cyclophosphamide.

*Steroid receptor assays*

During the operation an adequate sample of the tumour tissue was removed and immediately frozen in liquid nitrogen. Cytosol estrogen and progesterone receptor levels were determined at the Department of Clinical Chemistry, University of Oulu (Head: Prof. Reijo Vihko, M.D.) as previously described [14].

*Tissue preparation and DNA staining*

Two 30 μm sections were cut from the paraffin block from a site which contained over 10% malignant cells as verified histologically from a thin slice prepared between the two thick sections. Excessively necrotic and haemorrhagic areas were excluded. The methodology for the preparation of the paraffin sections for flow cytometry resembles that of Hedley *et al.* [15] with some modifications. The sections were deparaffinized by two washes in xylene, rehydrated in a sequence of 100, 95, 70 and 50% ethanol and washed in distilled water. The sections were then digested overnight with 0.25% trypsin (Orion Diagnostica, Helsinki, Finland) in a 10 mM Tris–HCl buffer at pH 7.5 (Sigma 7-9, Sigma Chemical Corporation, St Louis, U.S.A.) with 1 mM Na/EDTA (Titriplex, Merck, Darmstadt, F.R.G.) and 0.3% nonionic detergent Nonidet P40 (BDH Chemical Ltd., Poole, England). Undigested tissue fragments were removed and the liberated cells centrifuged and stained with 50 μg/ml ethidium bromide (Sigma) in Tris–HCl buffer in an ice bath for 15 min. Ribonuclease A I, 100 μg/ml, (Sigma) was added and the samples incubated for an additional 15 min at room temperature, after which the cells were filtered through a nylon net (pore size 55 μm).

*Flow cytometry*

Ten thousand ethidium bromide stained cells were run in an EPICS C flow cytometer (Coulter Electronics Incorporation, Hialeah, USA). Light scatter gates were established for every sample to reduce the signals from cell debris. The red fluorescence (> 610 nm) of the stained cells was collected and processed for storage and display. The electronics of the instrument were adjusted so that the fluorescence peak of cells having a diploid DNA content had an approximate channel No. 50. The slight variation in staining intensity from sample to sample made the use of internal ploidy standards impractical. The first DNA peak observed was classified as diploid and subsequent peaks as aneuploid. Diploid distributions with a G2/M fraction larger than 20% of the total cell count were classified as aneuploid. The percentages of cells in the various phases of the cell cycle were analysed with the Coulter software programme Statpack, using the rectangular histogram analysis method described by Baisch *et al.* [16]. The cell cycle distribution was not analysed in cases with few aneuploid cells or when large amounts of cell debris interfered with the determination of S-phase fraction.

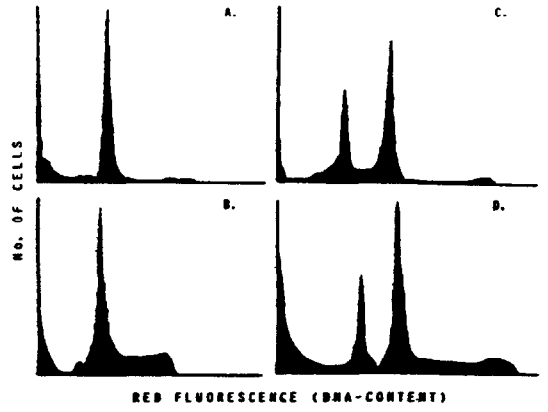


*Fig. 1. Representative DNA histograms from paraffin-embedded breast cancer samples. A and B consist of a single diploid DNA peak, whereas C and D consist of two peaks: diploid cells and aneuploid tumour cells. Peak CV:s are < 5.0%.*

*Statistics*

BMDP programmes were run in a DEC 10/20 computer at the University of Tampere for the statistical analyses of the material. BMDP1L was used in calculating the univariate product limit survival of the patient groups [17]. After ascertaining that deaths from other causes than cancer did not differ between the patient groups analysed, only cancer deaths were included in survival analysis. Deaths from other causes were treated as censored.

## RESULTS

The preparation of paraffin-embedded tumour slices for flow cytometry resulted in good DNA histograms in 92 of the 93 cases studied (Fig. 1). The mean coefficient of variation of the diploid DNA peak was 5.3 (range 3.4–8.2). The DNA indices were concentrated around the diploid DNA content and in the triploid–tetraploid region with 60% of the samples being aneuploid.

Ploidy of the tumour cells was not associated with the age of the patients or their menopausal status. The proportion of aneuploid tumours

*Table 1. Association of estrogen (ER) and progesterone receptor (PR) status with ploidy and S-phase fraction (SPF) of breast cancer*

| | Aneuploid (No./total) | | Aneuploid (%) | SPF (Mean ± SD) | |
|---|---|---|---|---|---|
| ER + | 33/60 | | 55 | 8.43 ± 4.72 | ** |
| ER − | 22/32 | | 69 | 11.05 ± 5.27 | |
| PR + | 34/65 | * | 52 | 7.98 ± 4.34 | *** |
| PR − | 21/26 | | 81 | 12.59 ± 5.38 | |

*$P < 0.05$ (Pearson $\chi^2$ test), **$P < 0.05$ (t-test), ***$P < 0.001$ (*t*-test).

*Table 2. Association of histological grade of the tumour with the ploidy and S-phase fraction of ductal infiltrating carcinoma of the breast*

| | Aneuploid (No./total) | Aneuploid (%) | SPF (Mean ± SD) |
|---|---|---|---|
| Grade I | 2/8 | 25 | 5.20 ± 3.54 |
| Grade II | 17/34 | 50 | 7.69 ± 3.46 |
| Grade III | 25/36* | 69 | 12.53 ± 5.24*** |

*$P < 0.05$ in Pearson $\chi^2$ test, ***$P < 0.0001$ variance analysis.

increased slightly with advancing stage from 9/20 (45%) in stage I to 32/52 (61%) in stage II and 12/20 (60%) in stage III–IV cancers. Aneuploidy tended to be more common in large as compared to small primary tumours as well as in axillary node positive as compared to node negative patients. These trends did not, however, reach statistical significance. Aneuploid tumours were more common in steroid, especially progesterone, receptor negative tumours as compared to positive ones (Table 1). Poorly differentiated i.e. grade III tumours were more often aneuploid than grade II or grade I tumours (Table 2).

The SPF of the tumour cells could be determined in 59 cases. SPF was significantly higher in aneuploid (14.12 ± 5.80%) than in diploid (6.58 ± 2.97%) cancers ($P < 0.001$). The SPF values of aneuploid tumours had a bimodal distribution, whereas those of diploid tumours had unimodal distribution (Fig. 2). SPF of the tumour cells was not significantly different in pre- and postmenopausal patients. SPF was also only weakly associated with the stage of the patients. Mean SPF increased from 8.90% in stage I to 10.63% in stage III–IV cancers.

Mean SPF was higher in steroid receptor-positive as compared to -negative tumours (Table 1). Especially the PR status was related to the SPF of the tumour cells. The mean SPF was 2.5 times higher in poorly differentiated than in well differentiated breast cancers (Table 2).

The disease-free interval and survival of patients with stage II–III tumours was analysed according

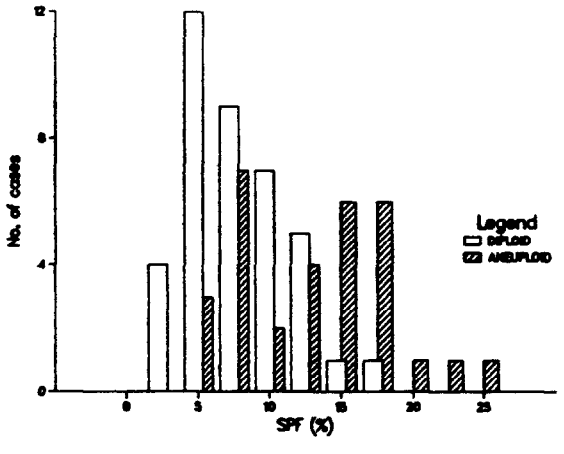


*Fig. 2. S-phase fraction (SPF) of breast cancer cells according to cellular DNA content.*

to the ploidy or the SPF of the tumour. The recurrence rate was almost two times lower in patients with diploid as compared to aneuploid tumours (Fig. 3). The survival of patients with diploid tumours was also slightly longer than that of patients with aneuploid tumours (Fig. 4).

The prognosis of the patients was more strongly associated with the SPF than the ploidy of the tumour cells. Patients with above median (> 7.5%) tumour SPF had significantly ($P < 0.01$) shorter disease-free interval (Fig. 5) as well as survival (Fig. 6) than those with below median SPF. Three out of 23 patients (13%) with low SPF died of breast cancer as compared to 13 out of 23 patients (56%) with high SPF.

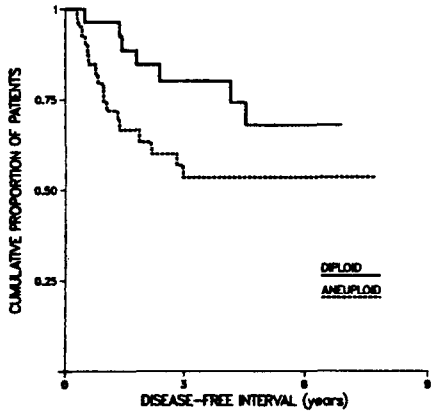


*Fig. 3. Residue-free interval in stage II–III breast cancer patients with either diploid* (n = 27) *or aneuploid* (n = 41) *tumours.* P = 0.05 *(Generalized Wilcoxon–Breslow test).*

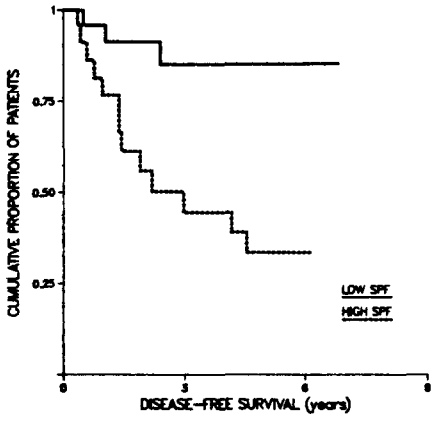


*Fig. 5. Residue-free interval in stage II–III breast cancer patients with below (low SPF,* n = 24) *and above (high SPF,* n = 24) *median tumour S-phase fraction.* P = 0.005 *(Generalized Wilcoxon–Breslow test).*

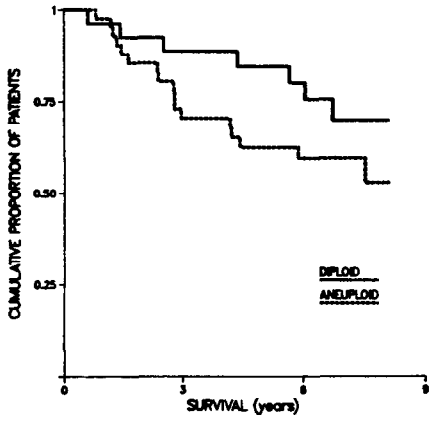


*Fig. 4. Survival of stage II–III breast cancer patients with either diploid* (n = 27) *or aneuploid* (n = 41) *tumours.* P = 0.15 *(Generalized Wilcoxon–Breslow test). Median survival time* 5.90 *years. Number of patients at risk at* 7.5 *years:* 3 *of diploid and* 7 *of aneuploid tumours.*

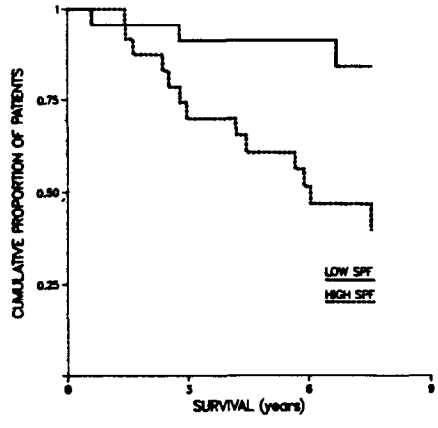


*Fig. 6. Survival of stage II–III breast cancer patients with below (low SPF,* n = 24) *and above (high SPF,* n = 24) *median tumour S-phase fraction.* P = 0.01 *(Generalized Wilcoxon–Breslow test). Median survival time* 6.16 *years. Number of patients at risk at* 7.5 *years:* 4 *of low SPF and* 2 *of high SPF groups.*

## DISCUSSION

The flow cytometric assay of SPF determines the proportion of cells having a DNA content between the G0/G1 and the G2/M compartments of the cell cycle. It does not give information on the actual rate of cell proliferation. In aneuploid tumours with two DNA peaks overlapping each others S-phases, the accurate determination of SPF is often difficult. The use of paraffin sections and enzymatic cell dissociation may produce cleaved nuclei, which increase the background debris signals and may interfere with the measurement of samples with low SPF. However, the amount of debris signals can be reduced by proper light scatter gates or by using thick 30 μm sections. Despite the methodological difficulties, the determination of SPF has been widely used in studies of tumour cell kinetics [5, 7, 8, 18, 19]. In the present work we analysed the SPF in only about 65% of the samples to obtain most accurate results. We used rectangular instead of Gaussian models for the estimation of the SPF of tumour cells. The rectangular model, developed by Baisch *et al.* [16], gives more reliable estimation of SPF in slowly proliferating cell populations than the Gaussian methods [7]. The median SPF in our material was 7.5%, which is very close to the value (7.1%) recently reported by McDivitt and coworkers [7] from an analysis of 168 primary breast tumours using comparable histogram analyses. These authors also obtained a good correlation between the thymidine-labelling index (TLI), a kinetic parameter of cell growth, and the flow cytometric estimate of SPF.

A low proportion of tumour cells in the S-phase of the cell cycle was associated with a low rate of disease recurrence and better survival in stage II–III breast cancer patients. The survival probability at 7.5 years was 85% in patients with low SPF and 40% in those with high SPF. Several studies have demonstrated a similar association of

TLI and the prognosis of breast cancer patients [3, 4]. Tubiana and coworkers [3] reported that the 10-year survival of patients with low TLI was 80% and that of high TLI 50%.

High SPF was most frequently observed in steroid receptor-negative and poorly differentiated tumours. The correlation of SPF with the stage of the disease was weak. These results are compatible with previous flow cytometric studies of breast cancer [7, 8, 18, 19]. However, in our study the SPF of PR positive tumours was significantly lower than that of PR negative ones, whereas a similar trend for ER status did not reach statistical significance. The results of McDivitt *et al.* [7] suggested that ER rather than PR status is related to the size of SPF. Heterogeneity of receptor expression in different parts of the tumour is a likely explanation for the discrepancy.

It has been observed that the TLI is a better predictor of prognosis than steroid receptor status [4], primary tumour size, histological grading or the lymph node status [3]. As a determinant of the proliferative rate the SPF could also be regarded as a primary prognostic indicator. However, only multivariate survival analysis in a larger patient material will reveal the prognostic weight of SPF as compared to conventional prognostic indicators.

Aneuploidy was weakly associated with advanced disease stage, and more strongly with the absence of steroid, especially progesterone, receptors and a poor differentiation state of the tumours, confirming previous studies on the subject [6–9, 11, 19]. The correlations of ploidy with the steroid receptor status or the stage of the cancer appear to be weak as they have not reached significance in all previous studies [6, 8]. The association of ploidy with the degree of cellular differentiation appears to be more strongly documented [7, 8, 19].

The present as well as previous results [6–8, 19] clearly demonstrate that the SPF is higher in aneuploid as compared to diploid tumours. A few initial studies have suggested that the rate of relapse is greater in aneuploid than in diploid tumours [9, 10, 11]. The present results, which are based on longer follow-up periods, confirm that the probability of relapse is greater in aneuploid than in diploid cancers. However, the survival of the patients in these two groups was not significantly different. It thus appears that the ploidy of the tumour cells is a less important prognostic factor than the SPF. Tumours with high SPF appear to be aggressive whether they are diploid or aneuploid. To obtain more information on the interrelationships of the DNA-parameters we are currently investigating the prognostic effect of SPF separately in diploid and aneuploid tumours in a larger patient material.

**Acknowledgements**—We thank Mrs. Leena Pankko and Ms. Eeva Särkkä for excellent technical assistance. This work was supported by grants from the Finnish Cancer Society and the Medica Research Foundation.

## REFERENCES

1. Atkin NB. Modal deoxyribonucleic acid value and survival in carcinoma of the breast. *Br Med J* 1972, **1**, 271–272.
2. Auer G, Eriksson E, Azavedo E, Caspersson T, Wallgren A. Prognostic significance of nuclear DNA content in mammary adenocarcinomas in humans. *Cancer Res* 1984, **44**, 394–396.
3. Tubiana M, Pejovic MH, Chavaudra N, Contesso G, Malaise EP. The long-term prognostic significance of the thymidine labelling index in breast cancer. *Int J Cancer* 1984, **33**, 441–445.
4. Meyer JS, Friedman E, McCrate MM, Bauer WC. Prediction of early course of breast carcinoma by thymidine labeling. *Cancer* 1983, **51**, 1879–1886.
5. Barlogie B, Raber MN, Schumann J *et al.* Flow cytometry in clinical cancer research. *Cancer Res* 1983, **43**, 3982–3997.
6. Hedley DW, Friedlander ML, Taylor IW. Application of DNA flow cytometry to paraffin-embedded archival material for the study of aneuploidy and its clinical significance. *Cytometry* 1985, **6**, 327–333.
7. McDivitt RW, Stone KR, Craig RB, Palmer JO, Meyer JS, Bauer WC. A proposed classification of breast cancer based on kinetic information. *Cancer* 1986, **57**, 269–276.
8. McGuire WL, Dressler LG. Emerging impact of flow cytometry in predicting recurrence and survival in breast cancer patients. *J Natl Cancer Inst* 1985, **75**, 405–410.
9. Ewers S-B, Långström E, Baldetorp B, Killander D. Flow-cytometric DNA analysis in primary breast carcinomas and clinicopathological correlations. *Cytometry* 1984, **5**, 408–419.
10. Coulson PB, Thornthwaite JT, Wooley TW *et al.* Prognostic indicators including DNA histogram type, receptor content, and staging related to human breast cancer survival. *Cancer Res* 1984, **44**, 4187–4196.
11. Hedley DW, Rugg CA, Ng ABP, Taylor IW. Influence of cellular DNA content on disease-free survival of stage II breast cancer patients. *Cancer Res* 1984, **44**, 5395–5398.
12. Scarff RW, Torloni H. *Histological Typing of Breast Tumours.* No. 2, Geneva, World Health Organization, 1968, 9–20.

13. UICC International Union Against Cancer. *TNM Classification of Malignant Tumours.* Third Edition, Geneva, 1978.
14. Vihko R, Jänne O, Kontula K, Syrjälä P. Female sex steroid receptor status in primary and metastatic breast carcinoma and its relationship to serum steroid and peptide hormone levels. *Int J Cancer* 1980, **26**, 13–21.
15. Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA. Method for analysis of cellular DNA content in paraffin-embedded pathological material using flow cytometry. *J Histochem Cytochem* 1983, **31**, 1333–1335.
16. Baisch H, Gohde W, Linden WA. Analysis of PCP-data to determine the fraction of cells in the various phases of the cell cycle. *Radiat Environ Biophys* 1975, **12**, 31–39.
17. Dixon WJ. *BMDP Statistical Software 1981.* University of California Press, Berkeley, California, U.S.A. 1981.
18. Olszewski W, Darzynkiewicz Z, Rosen PP, Schwartz MK, Melamed MR. Flow cytometry of breast carcinoma: II. Relation of tumour cell cycle distribution to histology and estrogen receptor. *Cancer* 1981, **48**, 985–988.
19. Moran RE, Black MM, Alpert L, Straus MJ. Correlation of cell-cycle kinetics, hormone receptors, histopathology, and nodal status in human breast cancer. *Cancer* 1984, **54**, 1586–1590.