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Original Paper

Prognostic Value of Quantitative Cytometry in a Series of 415 T1T2/N0N1/M0 Breast Cancer Patients—Preliminary Results

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Identifying prognostic markers in local regional breast carcinomas remains an important challenge today. DNA content obtained by flow cytometry, has been found to be of prognostic value; results with other methods remain less clear. This report describes DNA image cytometry patterns which are assessed with respect to disease-free survival. From June 1982 to December 1992, 415 patients under 75 years of age, without any previous or synchronous carcinoma, suffering from an invasive breast cancer classified as T1 (52.8%), T2 (47.2%), N0 (65.1%) N1 (34.9%), MO according to clinical TNM staging, were enrolled in this study. The median age was 53 (28–75) and 58.8% of the patients were premenopausal; 85.3% underwent a breast conservative procedure and 14.7% a modified radical mastectomy followed by postoperative irradiation. Histological axillary lymph node status, Scarff-Bloom grade and/or cytological grade and, oestrogen receptor content were used in decision-making for adjuvant treatment: hormonotherapy (48%) or chemotherapy (18.8%). Imprints were taken from the macroscopically visible lesion at the time of surgery, and a Feulgen staining was carried out on air dried smears to be analysed using the Samba 200 cell image processor (Alcatel TITN, France). Five parameters were systematically assessed: proliferation index; DNA histogram, integrated optical density, DNA malignancy grade, ploidy balance. With a median follow-up of 36 months (0–105), proliferation index ($P = 0.0008$), DNA histogram ($P = 0.0017$), integrated optical density (IOD) ($P = 0.018$) and DNA malignancy grade ($P = 0.017$) had a significant prognostic value on disease-free survival estimated by the Kaplan–Meier method. When these parameters were included in a Cox proportional regression hazards model, PR ($P = 0.01$), Scarff-Bloom histological grading ($P = 0.02$), axillary clearance ($P = 0.04$) were significant; however, in the same model, taking into account the axillary lymph node histological status, IOD was significant for pN– patients ($P = 0.03$), and proliferation index ($P = 0.03$) was significant for pN+. Such results need to be updated with a longer median follow-up, but they suggest that the mean DNA content, as measured by the integrated optical density (IOD), should be considered when deciding on medical adjuvant treatment with respect to patients with a negative axillary clearance.

Key words: breast cancer, prognostic factors, DNA image cytometry
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INTRODUCTION

Due to individual or mass screening programmes, breast carcinoma is increasingly diagnosed at the T1/T2, N0 clinical stage. If locoregional treatment is to be well managed by conservative treatment, clinicians have to foresee the potential behaviour of each patient's tumour to discriminate between low-risk metastatic breast carcinomas, which do not need systemic medical treatment, and high-risk tumours, which do. To implement such a policy, several prognostic factors are used, including indicators of tumour differentiation, growth rate, tumour aggressiveness or metastatic potential. Some of these are well established, validated and currently used; others are under investigation [1, 2]. Since 1982, the prognostic factors we have been using for medical treatment indications are Scarff–Bloom histological grading, cytological grade, histological lymph node axillary status, and steroid receptor content [3]. From a clinical research point of view, we are still

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evaluating static cytophotometry [4]. Flow cytometric studies of the DNA content of breast cancer cells have been reviewed and the consensus was that the percentage of cells in S phase is an important prognostic factor [5], but the issue of whether parameters from DNA image cytometry are independent prognostic factors is less clear and not all studies are in agreement [6–8]. The aim of this study was to evaluate the prognostic value of some of the parameters, defined by quantitative microscopy using Feulgen-stained imprints, on disease-free survival in an homogenous series of 415 invasive breast cancers clinically classified as T1/T2, N0/N1, M0 (UICC).

PATIENTS AND METHODS

Patients

The patient sample comprised 415 women, without previous or synchronous cancer, having an invasive breast carcinoma, classified as T1T2/N0N1/M0 (UICC classifications 1983 and 1987) studied from 1982 to 1992 (Table 1); the median age was 53 years (28–75); 58.8% were postmenopausal. They were treated in the Grenoble University Hospital or the Clinique du Mail.

Table 1. Patients' characteristics

Variable	Patients (n=415)	(%)
Age		
Median age (years)	53	
Mean age (years)	53.2	
Range	28–75	
Menopausal status		
Premenopausal	244	58.8
Postmenopausal	171	41.2
Stage		
T1	219	52.8
T2	196	47.2
N0	270	65.1
N1	145	34.9
pT1	254	61.2
pT2	161	38.8
pN N–	242	58.3
pN N ≤ 3	107	25.8
pN 3 < N ≤ 8	41	9.9
pN N > 8	25	6.0
Grade		
Scarff–Bloom grade = 1	123	29.6
Scarff–Bloom grade = 2	154	37.1
Scarff–Bloom grade = 3	114	27.5
Scarff–Bloom grade = NA*	24	5.8
Cytological grade 1†	84	21.3
Cytological grade 2†	163	41.3
Cytological grade 3†	148	37.4
Steroid receptor status		
ER–	114	27.5
ER+	301	72.5
PR–	162	39.0
PR+	253	61.0
Treatment		
Conservative surgery	354	85.3
Radical surgery	61	14.7
Postoperative irradiation	415	100
Hormonotherapy = yes	199	48.0
Hormonotherapy = no	216	52.0
Chemotherapy = yes	78	18.8
Chemotherapy = no	337	81.2

*Not assessed, †n = 395.

Clinical work up

Chest X-ray was mandatory; liver ultrasonography and bone scan were not mandatory but recommended in cases of positive axillary clearance ≥ 3.

Locoregional treatment

Conservative treatment by tumorectomy, sectoriectomy or quadrantectomy with axillary lymph node dissection was carried out in 85.3% of cases and radical mastectomy in 14.7% (Table 1). Irradiation of the mammary gland with telecobalt 60 or 6 MV photons up to 50 Gy was constant, most often with a boost to the surgical bed. Regarding regional treatment, no axillary irradiation was performed in case of negative lymph node dissection or involvement of less than 4 nodes of axilla; irradiation of the homolateral mammary chain was performed in case of central or internal quadrant tumour or if the number of positive axillary lymph nodes was ≥ 3.

Histological axillary lymph node status

After axillary clearance, yielding an average of 12 nodes, 58.3% were N– and 41.7% were N+.

Histopathological and cytological gradings

Histopathological Scarff–Bloom grade was established according to established criteria [9] and included all invasive carcinomas except some special types [10]. The cytological grade, according to Mouriquand and coworkers [11], was determined on imprints taken from the macroscopically visible lesion at the time of surgery in 395 cases. In case of galactophoric carcinoma, the highest grade (either histological or cytological) was chosen for medical treatment indication. Grade determinations were not duplicated so no estimation of intra- or interlaboratory reproducibility was possible.

Image cytometry

If cell image analyses are to be objective and reproducible, a precise definition of a protocol for sampling, fixation and staining is needed [12]. All samples were carefully prepared and analysed in the same manner. Imprints were air-dried and then fixed in a phosphate-buffered formalin (9%) acetone (45%) solution for 30 sec at 20°C (pH = 5.8). The Feulgen reaction was performed under the following conditions: hydrolysis by 6 N HCl for 60 min at 20°C followed by Schiff's reagent for 60 min at 20°C. Air-drying was used instead of immediate wet fixation because densitometric measurements were shown to be more reliable when Feulgen staining was carried out on air-dried smears [13].

Feulgen-stained nuclear images were analysed using the Samba 200 cell image processor (Alcatel TITN, France). The organisation, hardware and software packages of this system have already been described [14]. It takes approximately 5–15 min, according to smear cellularity, to analyse each imprint. All nuclei in smear fields, randomly selected by the observer, were analysed at 1000×. For each imprinted slide, 10–20 fields and 200–300 nuclei were analysed. Lymphocyte, fibroblast and macrophage nuclei were visually diagnosed and discarded. The objective description of the Feulgen-stained nuclei was obtained by the computation of extractable information related to geometry, densitometry and texture. Exter-

nal standard cells (trout erythrocytes) were used as staining quality control and as a DNA diploid (2c) reference. Between 50 and 100 nuclei were measured. For integrated optical density (IOD), the choice of an external trout erythrocyte DNA standard must be used with caution since it introduces negative errors in densitometric measurements, due to the real variations of DNA content between both species and to the trout's highly condensed chromatin. A correction factor (1.75) is used in order to bring the trout erythrocyte to the values of human normal diploid epithelial cells. A frequency histogram of DNA content in 'c' units was made for each slide: 'c' is defined as half the DNA content of the human normal diploid cells. Histograms were classified according to Auer and coworkers [15] into four classes. In addition, other parameters describing the DNA distribution in each cell population were computed from the DNA histogram: DNA malignancy grade (DNA-MG) according to Böcking and colleagues [16], ploidy balance and proliferation index according to Opfermann and coworkers [17]. Ploidy balance is calculated as the percentage of euploid cells (2c, 4c, 8c). This difference can vary from +100% (all cells are euploid) to −100% (all cells are aneuploid). Proliferation index is the percentage of cells outside the major peaks (regardless of its ploidy level) and the related peaks (peaks standing at half the modal value and/or at double the modal value). Five c exceeding rate (% of tumour cells with DNA content above 5c limit) has been excluded from the evaluation because this parameter was very closely correlated to IOD and DNA-MG ($P = 0.84$). The reproducibility of the measurements is good, as the coefficient of variation of IOD, after 20 measures of the same specimen, was found to be 5.24%.

Oestrogen receptor (ER) and progesterone receptor (PR) assays

ER and PR were assayed on cytosolic fractions. ER was evaluated using the Abbot monoclonal enzyme-immuno-assay kit [18]. PR was determined using 3H-ORG 2058 (Amersham, U.K.) as the ligand, and the dextran-coated charcoal procedure. The data were processed by Scatchard plot analysis. ER and PR data from the laboratory are included in the interlaboratory quality control of the EORTC and French Centres for the Fight against Cancer. Tumours with oestrogen and progesterone receptor levels higher than 10 fmoles of protein were considered positive [19].

Adjuvant medical treatment.

The following schedule was followed:

(i) N−, grade I/II, ER+: no medical adjuvant treatment;
(ii) ER+, with only one poor prognostic factor (grade III, N+ ≤ 3): tamoxifen, 20 mg per day for 2 years;
(iii) two poor prognostic factors out of three (grade III, N+≥ 3, RE−): six cycles of CMF or FAC.

Adjuvant hormonotherapy was proposed for 48% of the cases and adjuvant chemotherapy was used in 18.8%.

Follow-up

Follow-up was carried out by radio-oncologists and surgeons. Lung radiography was conducted every 12 months, and mammography every 12–18 months according to the surgical treatment. Local and/or regional recurrence were all histologically confirmed. Metastasis were diagnosed by lung radiography, tomodensitometry, echography or bone scintigraphy, according to their location. Positive Ca 15-3 was not considered as a recurrence criterium.

Statistics

Data were registered with a four dimension software. Details of patients' follow-up were obtained from the inpatient or outpatient records, or by writing to the attending surgeon or general practitioner. At the point date (17 November 1994), the median follow-up was 36 months (0–105). We expressed the results according to the disease-free survival with a confidence interval of 95%, taking the date of surgery as a reference. The monofactorial analysis of the cytometrical parameters took account of their median value or, if necessary, a cut-off value. Disease-free survivals were computed according to Kaplan–Meier [20] and compared using the log-rank test and the Cox proportional hazards regression model [21]. All calculations were performed using S+ software.

RESULTS

At the time of analysis, 304 women were alive free of disease, 50 were alive with cancer, 40 were lost to follow-up, 18 died of cancer and 3 died of complications pertinent to cancer. There were 52 relapses: 33 metastases and 21 local and/or regional relapses (2 patients had both metastatic and local relapse). Disease-free survival rates are shown in Table 2 according to each parameter. Table 3 shows the results of pathological, cytometrical and biochemical parameters. The proliferation index appears to be the best prognostic factor ($P = 0.0008$), followed by DNA histogram ($P = 0.0017$), IOD ($P = 0.018$) and DNA malignancy grade ($P = 0.017$). Conversely, there was only a trend for cytological grade ($P = 0.07$). As expected, Scarff–Bloom grade ($P = 3.7 \times 10^{-5}$), PR

Table 2. 5-year disease-free survival

Variable	Number of patients	5-year survival	(%) CI
IOD < 4000	124	83	72–97
IOD ≥ 4000	291	77	70–84
DNA-MG < 0.53	204	84	75–94
DNA-MG ≥ 0.53	199	75	67–84
PI < 1.78	209	86	78–95
PI ≥ 1.78	206	72	63–82
PB < 0	226	78	69–87
PB ≥ 0	189	81	74–90
HISG=1.2.5	171	85	75–97
HISG=3.4	220	76	68–84
Scarff–Bloom grade=1	123	94	88–100
Scarff–Bloom grade=2	154	75	65–87
Scarff–Bloom grade=3	114	72	61–84
pT=1	254	85	78–93
pT=2	161	70	60–81
PR−	162	65	53–80
PR+	253	86	80–93
ER−	114	70	59–84
ER+	301	83	76–90
Axillary clearance (−)	242	83	75–92
Axillary clearance (+)	173	73	63–83
Cytological grade 1	84	92	85–99
Cytological grade 2	163	79	69–90
Cytological grade 3	148	72	61–84
Cytological grade 1/2	247	82	75–91

IOD, integrated optical density; DNA-MG, DNA malignancy grade; HISG, DNA histogram; PI, proliferation index; PB, ploidy balance; PR, progesterone receptor; ER, oestrogen receptor.

Table 3. Monofactorial analysis of pathological, cytological and biochemical parameters

Variable	Cut-off point	Chi-squared	*P* value
Scarff–Bloom grade	1/2/3	20	3.7×10^{-5}
Pathological tumoral diameter (cm)	1.8 (median)	17	3.6×10^{-5}
pT	1/2	8.7	0.0032
Axillary clearance	0 (median)	5.6	0.018
pN	0/1–3/3–8/> 8	15	0.002
Proliferation index	1.78 (median)	11	0.0008
DNA histogram	1.2.5/3.4	9.9	0.0017
Integrated optical density	4406 (median)	5.6	0.0180
DNA malignancy grade	0.53 (median)	5.7	0.0170
Cytological grade	1/2/3	5.3	0.07
Ploidy balance	0	0.1	NS
ER	Positive/negative	5.3	0.021
PR	Positive/negative	17	4.8×10^{-5}

NS, not significant.

($P = 4.8 \times 10^{-5}$), tumour diameter (3.6×10^{-5}), pT1 versus pT2 ($P = 0.0032$), positive nodes ($P = 0.018$), pN (0.002), ER ($P = 0.02$) are prognostic parameters in a monofactorial assessment.

We took into account the Cox model analysis with a step by step progression by entering the above pathological and biochemical parameters from the most to the least significant, then adding similarly the cytological features: PR ($P = 0.01$), Scarff–Bloom grade ($P = 0.02$), axillary clearance ($P = 0.04$) were the only significant prognostic parameters (Table 4). For the whole cohort, no cytological variables were significant: IOD ($P = 0.12$), HISG ($P = 0.11$), PI ($P = 0.24$), DNA-MG ($P = 0.30$). Cytological grading was not significant when histological grading was excluded from the Cox model, and PR was taken into account. Moreover, since approximately 48% of the cases had been given adjuvant hormonal treatment and 18.8% chemotherapy, we considered two cohorts in order to see if the variable treatment had a prognostic value: 1 of 185 patients without adjuvant treatment, and 1 of 230 patients who were given chemotherapy and/or hormonotherapy. When the variable treatment was introduced into the three models (whole cohort, pN0 cohort, pN+ cohort), it was not significant. Since 58.3% of the cases were pN0, we attempted to assess the prognostic value of cytometrical parameters separately, both for pN0 and pN+. Table 5 shows that for pN0, using a dichotomised value of pT (pT1/pT2), IOD (0.03) and pT (0.03) were significant. Table 6 shows that for pN+, PR ($P < 0.01$), Scarff–Bloom ($P = 0.022$) and PI ($P = 0.024$) use the only prognostic factors.

DISCUSSION

The patient cohort analysed had a median tumoral diameter of 1.8 cm, 58.3% of the cases were lymph node negative, 66.7% were Scarff–Bloom grade 1 or 2 and 72.5% had oestrogen receptor positive tumours. These clinical and pathological characteristics are in agreement with those obtained from breast cancer screening [22]. Indications for adjuvant treatment were based on histological and/or cytological grading, axillary lymph node pathological status and the biochemical evaluation of oestrogen receptors: hormonotherapy was used in 48% of the cases and chemotherapy in only 18.8%.

We included the cytological grading according to previous studies from Grenoble [11]; this grade was shown to be highly correlated with Scarff–Bloom grading. If disagreement occurred between both gradings, the highest was used. In our study, Scarff–Bloom grade, despite its lack of reproducibility

Table 4. Log-rank test and regression coefficient of the significant prognostic variables in the Cox model for relapse-free survival

Variable	Coefficient	S.E./Coefficient	Coefficient/S.E.	*P* value
PR	−0.79	0.31	6.58	0.01
Scarff–Bloom grade	0.55	0.23	5.66	0.02
Axillary clearance	0.077	0.037	4.24	0.04
PI	0.038	0.024	2.48	0.12

Table 5. Log-rank test and regression coefficient of the significant prognostic variables in the Cox model for relapse-free survival for negative axillary clearance

Variable	Coefficient	S.E./Coefficient	Coefficient/S.E.	*P* value
Pathological tumoral diameter	0.56	0.26	4.5	0.03
IOD	3.2×10^{-4}	1.5×10^{-4}	4.6	0.03

Table 6. Log-rank test and regression coefficient of the significant prognostic variables in the Cox model for relapse-free survival for positive axillary clearance

Variable	Coefficient	S.E./Coefficient	Coefficient/S.E.	*P* value
PR	−1.16	0.43	7.12	< 0.01
Scarff-Bloom grade	0.74	0.32	5.26	0.022
PI	0.065	0.029	5.08	0.024

[23], appears to be the best prognostic factor, rather than the histological analysis of axillary lymph nodes.

The cytological grade, although taking into account the topographical distribution of the cells, the cell size, the nuclear and nucleolar features, and mitosis [11], is slightly under the threshold of statistical significance in the monofactorial analysis. It was determined by the same pathologist and this obviates its lack of concordance estimated at 28% by Van Diest [24]. However, the cytological grading does not appear in the multifactorial analysis: this confirms the study of Ciatto and coworkers [25] on 213 cases (191 T1T2) when the cytological grade was evaluated on imprints according to Mouriquand's criteria.

The goal of quantitative image analysis is to obtain prognostic features which show good objectivity and reproducibility and are biologically relevant. In this study we choose to evaluate only five parameters expressing either the mean DNA content (IOD, ploidy index) or heterogeneity of the DNA quantity of the tumoral cell population (DNA histogram, DNA-MG). IOD is significant in the Cox model for the pN0 cohort ($P = 0.03$), whereas the Proliferation Index is powerful for the pN+ cohort ($P = 0.024$); in this latter case this cytological prognostic factor becomes less important given the known metastatic risk. These results are at variance with Fallanius and coworkers [6] who, using microspectrophotometry, studied 409 breast cancer patients (90% T1T2, 10% T3) with a follow-up ranging from 8 to 13 years after primary treatment. These authors demonstrated that the DNA histogram typing with a Cox regression gave significant prognostic information, as opposed to any other variables. These results were confirmed by von Rosen and coworkers [26]: in a study of 464 pT1pT2 they showed that both the Auer DNA profile ($P < 0.01$) and pathological tumour size ($P < 0.01$) had a significant prognostic value in node-negative patients; but for node positive patients, only pT remained significant ($P < 0.01$). For flow cytometry, on which most of the studies on the prognostic significance of DNA evaluation have been conducted, the percentage of cells in S-phase has emerged as an important prognostic factor when considered as a global indicator of disturbed proliferation for both node-negative and node-positive invasive breast cancer [5].

To conclude, a longer follow-up would enable us to assess the definitive evaluation of disease-free survival with respect to IOD; nevertheless this variable seems to be of particular interest for node-negative patients, suggesting that it ought to be taken into account when deciding on adjuvant treatment.

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