Eur J Cancer, Vol. 28, No. 2/3, pp. 504–507, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Feature Articles

Is DNA Flow Cytometry a Useful Investigation in Breast Cancer?

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INTRODUCTION

IN RECENT years there have been a large number of publications on DNA flow cytometry in breast cancer. Despite this a clear role has not been defined for this investigation in the management of individual patients. In this article, we review the published data in order to assess the impact of this technique on the estimation of the risk of recurrence, on selection of groups of patients for treatment, and on predicting response to systemic therapy.

Flow cytometry is a technique which can rapidly and quantitatively measure a wide variety of cellular constituents. The ability of flow cytometry to estimate cellular DNA content is based on the measurement of fluorescence from dyes which bind in a stoichiometric manner to DNA. Cellular DNA content is usually expressed as DNA ploidy, representing the DNA content of cells under investigation as a ratio to that of normal (diploid) controls. As the DNA content is duplicated prior to cell division, mathematical models have been derived which can estimate the percentage of cells in different phases of the cell cycle. In cancer research, interest has centred on the percentage of cells in the DNA synthetic phase of the cycle (SPF). DNA flow cytometry has the advantage that it can be performed on fresh, frozen or paraffin-embedded tissue. Although it involves a relatively high initial capital outlay, it is less labour intensive than other methods of assessing proliferation, e.g. measurement of the thymidine labelling index. Flow cytometry, however, requires physical disruption of tissues, and thus the relationship between proliferation and tumour morphology, which can be observed using immunohistochemical techniques, e.g. Ki67 or PCNA/cyclin staining, cannot be studied. Moreover, interpretable DNA histograms cannot be obtained from all tumour samples, and SPF cannot be estimated from all DNA histograms, e.g. in the presence of multiple or overlapping aneuploid populations of cells.

FLOW CYTOMETRY CHARACTERISTICS AND OTHER TUMOUR FEATURES

Many groups have published data examining the relationship between DNA ploidy or, less commonly, SPF and other recognised prognostic characteristics such as tumour size, extent of axillary lymph node involvement, histological grade and steroid hormone receptor status (Tables 1 and 2). No consistent relationship has been demonstrated between DNA ploidy and tumour size. While some authors have noted an increased incidence of aneuploidy in larger tumours [3, 9–11, 18], others have not [4–7, 12, 13, 17, 19]. Similarly, there is little evidence of an association between SPF and tumour size. Although the majority of published studies have not recorded a correlation between DNA ploidy and lymph node status [3, 4, 6, 7, 10, 11, 19, 20], a substantial minority of trials have reported an increased incidence of aneuploidy in node positive disease [12–18]. There is less evidence to support an association between SPF and lymph node status.

Table 1. Relationship between DNA ploidy and other prognostic variables

Reference	*n*	% AN	Nodes	Size	Grade	ER	PgR
1	92	92	NA	NA	+	+	NA
2	46	56	NA	NA	++	+	NA
3	638	60	0	++	NA	NA	NA
4	143	72	0	0	+	0	0
5	66	61	NA	0	++	0	NA
6	226	54	0	0	0	+	0
7	168	55	0	0	NA	0	0
8	145	57	NA	NA	NA	+	+
9	59	54	NA	++	+	0	NA
10	354	60	0	+	++	0	NA
11	65	48	0	+	NA	NA	NA
12	308	63	+	0	++	+	+
13	473	72	+	0	++	+	+
14	565	72	+	+	NA	0	NA
15	1184	57	+	NA	NA	++	++
16	300	62	++	NA	+	++	++
17	472	63	+	0	NA	++	NA
18	351	68	++	+	++	NA	NA
19	140	69	0	0	++	0	0
20	690	73	0	++	NA	++	NA

%AN = % aneuploid; 0 = $P > 0.05$; + = $0.01 < P < 0.05$; ++ = $P < 0.01$; NA = not assessed.
ER = oestrogen receptor, PgR = progesterone receptor.

The relationship between flow cytometry characteristics and histological type of breast carcinoma has only been analysed in a few centres. In general, invasive lobular, tubular, papillary and colloid carcinomas are more likely to be diploid, with a low SPF than invasive ductal tumours [16, 18], while medullary carcinomas are likely to have a high SPF. There appears to be a clear relationship between DNA ploidy, SPF and histological grade. Almost all reports note a strong correlation between high histological grade (i.e. poorly differentiated tumours) and both aneuploidy [1, 2, 4, 5, 9, 10, 12, 13, 16, 18, 19] and high SPF [5, 6, 7, 13, 16, 18, 21–23].

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Received 10 Sep. 1991; accepted 9 Oct. 1991.

Table 2. Relationship between SPF and other prognostic variables

Reference	*n*	Nodes	Size	Grade	ER	PgR
21	90	NA	0	+	+	NA
22	79	0	NA	++*	+	+
5	66	NA	NA	++	NA	NA
6	216	0	0	++	++	+
7	168	0	0	++	++	+
13	285	0	0	++	0	0
15	1084	+*	NA	NA	++	++
16	300	0	0	++	++	0
23	308	0	0	++	0	+
17	290	0	++	NA	++	NA
18	223	+	0	++	NA	NA
19	134	0	0	++	0	0

0 = $P > 0.05$: + = $0.01 < P < 0.05$; ++ = $P < 0.01$: NA = not assessed.
*Significant association within diploid tumours only.

Most investigators have found the presence of oestrogen or progesterone receptors in a tumour to be associated with a low SPF [6, 7, 15, 16, 17, 21, 22], although there is a considerable overlap between the SPF values of receptor positive and receptor negative tumours. While some authors have also noted an increased incidence of aneuploidy in the receptor negative group [1, 2, 6, 8, 12, 13, 15–17, 20], others have not [4, 5, 7, 9, 10, 19].

DNA PLOIDY, SPF AND PROGNOSIS

As flow cytometric characteristics appear to be related to other features of the tumour which can predict clinical outcome, the relationship between DNA ploidy, SPF and prognosis has been explored in a number of large retrospective studies in the past 5 years (Table 3). Most reports examine the ability of DNA ploidy or, less commonly, SPF to predict time to relapse and survival for patients with operable stage I–III breast cancer. On univariate analysis, there is evidence of an association between high SPF and shorter relapse free survival [13, 17, 19] and survival [17–19, 23, 25]. While patients with aneuploid tumours also tend to have a poorer prognosis than those with diploid tumours [12, 13, 17, 18, 20, 25], this difference does not always reach statistical significance [10, 19]. Of more interest, however, is whether flow cytometry provides information on prognosis which is independent of that provided by other, more commonly available, investigations. Multivariate analyses do not support the ability of DNA ploidy to provide independent prognostic information. When DNA ploidy was included as a variable in the Cox model, it was found to give significant prognostic information in only three studies [12, 14, 20], two of which did not include histologic grade as a variable [14, 20]. While SPF retains its impact on prognosis when tumour size, lymph node status and steroid hormone receptor status are included in the regression analysis [17, 23], independent prognostic significance is usually lost when the histological grade is included [13, 19]. The exception to this is a study with long term follow-up (median 28 years) from Finland, where both SPF and histologic grade gave independent prognostic information on survival [18].

Table 3. Prognostic significance of DNA ploidy and SPF: P values for univariate analysis

			Ploidy/DNA index		SPF	
Reference	*n*	Stage	RFS	OS	RFS	OS
12	297	I-III	NA	<0.001	NA	NA
13	565	I-IV	0.06*	0.04	NA	NA
10	354	A-C	NS	NS	NA	NA
13	473	II(N+)	0.03	0.02	<0.001	NA
23	308	I-III	NA	NA	NA	<0.05†
24	156	I-III	<0.005	NS	NA	NA
18	351	I-IV	NA	0.0001	NA	<0.0001
17	472	I-III	0.003‡	0.004	0.004‡	<0.0001
19	140	I,II	NS	NS	0.008	0.004
25	225	I,II	NA	0.015	NA	0.01
20	690	I-III	NA	0.02	NA	NA

NS = $P > 0.05$; NA = not assessed.
*Distant metastasis free survival.
†Diploid tumours, $P=0.002$; aneuploid tumours, $P=0.02$.
‡Significant only within first 2.5 years of follow-up.

In recent years there has been particular interest in defining prognostic factors for patients with node negative breast cancer. This has arisen because of the reports that adjuvant chemotherapy [26] or endocrine therapy [27] can improve prognosis for this group of patients. As up to 70% of patients with node negative disease are likely to remain free of disease following surgery alone, a number of studies have addressed the ability of flow cytometry characteristics to define the subgroup of patients at high risk of relapse. While aneuploidy was initially reported to be associated with a significantly poorer relapse free survival [28] and survival [29], this observation has not been confirmed in subsequent studies [30–34]. While SPF was originally reported to be of prognostic significance only for patients with diploid tumours [28, 32], subsequent results suggest that SPF can also discriminate between high and low risk of relapse for patients with aneuploid tumours [31, 35]. Four studies have reported that SPF is an independent prognostic factor [28, 29, 31, 32] in node negative breast cancer, while no independent effect was noted in two others [30, 33]. There is little evidence of an independent prognostic role for DNA ploidy. Prognostic models based on combinations of SPF and tumour size [31], SPF, ploidy and progesterone receptor status [28] or SPF, tumour size and progesterone receptor status [29] have been derived in an attempt to select a group of patients at high risk of relapse, who might be suitable candidates for adjuvant chemotherapy.

FLOW CYTOMETRY AND RESPONSE TO TREATMENT

While data continue to accumulate indicating that DNA flow cytometry may provide information useful in predicting recurrence and survival, the ability of DNA ploidy or SPF to predict response to systemic treatment (chemotherapy or endocrine therapy) is a relatively unexplored area. The few studies addressing this question can be subdivided based on the clinical setting in which systemic treatment was given. Of interest given the increasing use of preoperative chemotherapy for patients with large primary tumours, two French groups have published small studies examining the ability of DNA flow cytometry performed on fine needle aspirates from the primary tumour to predict chemosensitivity. Briffod *et al.* [36] noted a significantly higher objective response rate to combination chemotherapy in aneuploid tumours (15/25) than in diploid

tumours (1/10, $P = 0.008$). In contrast, Remvikos *et al.* [37] did not observe a significant difference in response to chemotherapy between the two ploidy groups. However, tumour responsiveness was significantly related to SPF, with all 12 patients who had SPF of 10% or more showing demonstrable tumour regression. While these results are interesting, larger, prospective studies are needed to decide whether DNA flow cytometry gives any clinically useful information.

In reality, the target cells of interest in early breast cancer are those of occult micrometastases rather than of the primary tumour. The relationship between ploidy and SPF in the primary tumour and response to adjuvant chemotherapy has also been assessed, although, in this situation, response can only be measured indirectly as a prolongation of the time to relapse. Hedley *et al.* [13] observed no apparent difference in the effects of ploidy or SPF on relapse free survival within the treatment arms for patients treated as part of the Ludwig Breast Cancer Studies I–IV of adjuvant chemotherapy and/or endocrine therapy for node positive breast cancer. Among patients with node positive disease entered into a randomised trial comparing patients given CMF with untreated controls, O'Reilly *et al.* [38] noted that chemotherapy benefitted premenopausal patients irrespective of whether tumours had low or high SPF. A preliminary report from Dressler *et al.* [39] on the impact of SPF on response for patients treated as part of the Intergroup trial of adjuvant chemotherapy for node negative disease suggests that chemotherapy shows a benefit compared to observation among patients with a high SPF, with insufficient information as yet to show an effect of treatment versus observation in the low SPF group.

The only study correlating DNA ploidy and SPF of the primary tumour to the response of metastatic disease to chemotherapy found no significant relationship [40]. Three studies have investigated the relationship between ploidy and response to endocrine treatment in advanced breast cancer. Stuart Harris *et al.* [41] using ploidy evaluated on tissue from the primary tumours or their metastases, reported no significant relationship between ploidy and response in 42 patients. Baildam *et al.* [42] observed a significantly better response to first line endocrine therapy in tetraploid and near tetraploid tumours than others, while Seymour *et al.* [43] reported in a small study that response to second line endocrine therapy was significantly higher in patients with diploid tumours.

MULTIPARAMETER FLOW CYTOMETRY

As the flow cytometer can measure several fluorescent probes simultaneously, a number of centres are now investigating the role of multiparameter flow cytometry in breast cancer. The combination of cell surface markers such as anti-keratin antibodies may help to produce histograms uncontaminated by stromal cells [44]. Additional information about the cell cycle may be obtained by the *in vivo* injection of bromodeoxyuridine (BrdU) some hours before biopsy of the tumour. This thymidine analogue is incorporated into cells during the S-phase of the cell cycle and can be detected on flow cytometric analysis using a fluorescence-labelled anti-BrdU monoclonal antibody. Using this technique, the rate of movement of cells through the cell cycle and the potential doubling time of the tumour can be measured as well as the labelling index [45]. Multiparameter analysis of proliferation-associated antigens such as PCNA/cyclin and Ki-67 [46] may give clinically useful information.

CONCLUSIONS

The accumulating evidence from a large number of studies supports the association between high SPF, and to a lesser extent aneuploidy, and an increased risk of recurrence and death for patients with breast cancer. This prognostic information appears to be independent of tumour size, nodal status and steroid hormone receptor status, but, due to the strong association between high SPF, aneuploidy and poor histological differentiation, is frequently reduced or abolished when tumour grade is included in the analysis. Can this information be useful in clinical practice? The most likely situation where flow cytometric measurements might influence patient management is in the selection of node negative patients for adjuvant chemotherapy. However, while flow cytometry is a more objective technique than the assessment of tumour grade, little has been done to standardise methods of flow cytometry analysis between different laboratories. Differences in criteria for diagnosing aneuploidy, in deciding whether and how to measure SPF in samples with an aneuploid population of cells, in the models used to calculate the SPF, and in the definition of what constitute a high SPF all make if difficult to apply results from individual centres in general practice. In addition, more information is needed on the ability of flow cytometric measurements to predict response to treatment, particularly in the adjuvant setting.

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