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Determining factors which predict response to primary medical therapy in breast cancer using a single fine needle aspirate with immunocytochemical staining and flow cytometry

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Abstract The increasing use of neoadjuvant chemotherapy and endocrine therapy in the management of breast cancer has lead us to evaluate and optimise the standard technique of cytocentrifugation of a single fine needle aspirate (FNA) taken from a breast tumour in-vivo, to determine a range of both immunocytochemical and flow cytometric factors which are predictive of response to primary medical therapy. Some of these factors are also of prognostic significance in early stage disease. An analysis of the cellularity and immunocytochemical staining characteristics of FNAs obtained from a series of 206 patients with palpable breast cancers indicate that in a sample of 46 cases it is possible to measure oestrogen receptor, progesterone receptor and c-*erb*B-2 providing over 400 cells per slide are obtained, with material obtained in a single FNA prepared by cytocentrifugation, using standard immunocytochemical methods. The staining results obtained were comparable to those obtained using frozen or paraffin embedded tissue sections taken from the same tumour. In addition an estimate of the proliferation indices could be made by flow cytometric analysis of the residual cell suspension fluid with measurement of DNA index and S-phase fraction in 131/164 (80%) and 110/164 (67%) of cases respectively. Providing all FNAs obtained for cytocentrifugation were taken at first presentation rather than immediately following a standard FNA, then it was possible to obtain adequately cellular (>400 cells/slide) samples in 96 out of 126 (75%) of the last cohort of breast aspirates. These effects may be independent of T stage but not histological type as patients with lobular tumours only produced cellular aspirates in 1/7 (14%) of cases. The advantages and disadvantages of using FNA over trucut biopsy are discussed further.

Key words Fine needle aspiration · Breast cancer · Immunocytochemistry · Cytospin technique · Flow cytometry

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Introduction

There has been considerable interest in recent years in the use of primary (neoadjuvant) chemotherapy or endocrine therapy in both operable and locally advanced breast cancer [3, 13, 15, 29].

For any such patient the determination of biological factors which may predict response to such treatment is of considerable value. These factors include oestrogen or progesterone receptor status which can be used to predict response to tamoxifen therapy [6, 7, 25]. Recent studies have suggested a relationship between c-*erb*B-2 expression and response to adjuvant chemotherapy in early stage breast carcinoma [17, 22]. The proliferation indices of the tumour (DNA index and S-phase fraction) have also been shown to be predictive of response to neoadjuvant chemotherapy in locally advanced disease [23]. However when treating patients by primary medical therapy, with the tumour remaining in-vivo, it is necessary to determine these factors without causing undue trauma to either patient or tumour. The two methods presently available for obtaining samples for such analyses are trucut biopsy or fine needle aspiration (FNA).

The present study seeks to determine the feasibility of using a single FNA to determine not only cytodiagnosis but also a series of immunocytochemical (oestrogen receptor [ER], progesterone receptor [PgR] and the c-*erb*B-2 gene product) and flow cytometric (S-phase fraction [SPF] and DNA index [DI]) factors which may be of importance in terms of both prognosis as well as an indicator or response to primary medical therapy.

Using the technique of cytocentrifugation which has previously been described [9, 21] we have attempted to determine factors which may maximise or improve the standard technique which has previously reported an efficiency rate of <50% in determining even cytodiagnosis [9].

Materials and methods

Two hundred and eleven successive patients presenting with a palpable breast lumps were included into the study which was conducted between October 1990 and October 1992. Only patients in whom the clinical and mammographic evidence pointed to the lesion being a probable carcinoma were included. Two hundred and six of these were eventually found to be carcinoma (Invasive or ductal carcinoma in situ [4 cases only]).

The distribution of patients according to breast tumour size as defined by the UICC T-stage classification is shown in Table 1.

All fine needle aspirates were taken using a blue 23 gauge needle and a 10 ml syringe. Two needle aspirates were taken from each tumour. One of the aspirates was handled as a standard diagnostic aspirate, the contents of which were smeared onto two slides. The other aspirate was flushed with 2 ml of Minimum Essential Medium (MEM) with phenol red and 25 mM HEPES buffer to form a cell suspension. This suspension was then prepared by the method shown below to make up to twelve slides suitable for immunocytochemical analysis.

In the first 83 cases (Group 1) the sample taken as the standard diagnostic aspirate was always taken first and was then immediately followed within 10 min by the sample for cytocentrifugation. In the subsequent 123 cases (Group 2) the samples taken for cytocentrifugation were taken first if at all possible. But in the event that a diagnostic aspirate had been taken before the patient was referred to the unit, the policy was not to repeat any aspirate until a gap of at least 1 week had passed from the time of the initial FNA.

In a series of 32 patients from Group 2, we further investigated whether pre-filling the syringe with MEM before aspiration in any way improved the cellularity of the cytospin preparations. This was done by taking two aspirates one week part from the same tumour with initially a needle and syringe containing 0.2 ml of MEM ('wet' needle). A repeat aspirate was taken 1 week later from the same tumour, by the same aspirator, with a dry needle and syringe.

For the preparation of cytospin slides from cell suspension, 100 µl of the cell suspension fluid was pipetted into each of eleven Shandon cytospin chambers with a further 200 µl of tissue culture medium. These were then centrifuged at 500 r.p.m. for 5 min onto 3-aminopropyltriethoxsilane (APES) coated slides. The remaining 0.9 ml of suspension fluid was snap frozen in liquid nitrogen and used for flow cytometry (see below). The slides were air dried for 10–15 min at room temperature. Five of these were stored unfixed at −80° C while the six remaining slides were immediately prefixed before storage in 50/50 methanol and acetone solution at −20° C. One of these slides was stained with May-Grunwald-Giemsa and scored for both the degree of malignancy (see Table 2) and cellularity of the specimen (see Table 3). The scoring was performed by the same Consultant Cytopathologist to maintain consistency of assessment.

Table 1 Distribution of cytospin samples in relation to T-stage of the tumour in all pretreatment samples

Stage	Total
T1/T2	172 (84%)
T3/T4	26 (12%)
Lymph node	8 (4%)
Total	206

Table 2 Cytological grading of fine needle aspirates as used at the Royal Marsden Hospital

C5	Carcinoma
C4	Suspicious, probably malignant
C3	Atypical, probably benign
C2	Benign
C0	Inadequate, acellular, insufficient for diagnosis

Table 3 Scoring system for the cellularity of FNAs as used at the Royal Marsden Hospital

Score	Cellularity	Approx. No. of cells per slide
C5/+++	Cellular	>400 Malignant Cells
C5/++	Moderately cellular	50–400 Malignant Cells
C5/+	Scanty	<50 Malignant Cells
C2/+	Scanty	Only benign duct epithelial cells

Any patient who did not get a definite cytodiagnosis (scored as C5) on either the standard FNA or cytospin preparation had a trucut biopsy. If this was also negative an excision biopsy of the tumour was performed.

Immunocytochemical staining for ER, PgR and c-*erb*B-2 was performed on a selection of 46 cases. Twenty of these had been scored as C5/+++ with over 400 malignant cells/slide. The remaining 26 cases had been scored as only C5/++ (see Table 3). ER and PgR immunostaining was performed using the primary rat monoclonal antibodies (H222 and KD68 respectively) as provided by Abbott with an immunoperoxidase technique using the standard kit as supplied [6]. C-*erb*B-2 staining was also performed using a standard technique with ICR-12, a rat monoclonal antibody as has previously been described [33]. In the 20 cases scored as C5/+++, frozen (for ER and PgR) and paraffin embedded (for c-*erb*B-2) tissue sections taken from the same tumour after surgery were also immunostained so that a comparison between this result and that obtained by FNA and cytocentrifugation could be made.

For flow cytometric analysis all the reagents were purchased from Sigma (Poole, Dorset). The residual suspension fluid remaining (0.9 ml) was thawed out at 37° C, centrifuged at 1000 r.p.m. for 15 min and the pellets resuspended in 200 µl of a stain-detergent solution consisting of 1 g trisodium citrate, 564 mg sodium chloride, 300 µl Nonidet P-40, 10 mg propidium iodide in 1 L of distilled water. To this suspension of nuclei, 20 µl of a 1 mg/ml solution of RNase was added and the suspension kept on ice for 30 min before analysis.

The nuclei were analysed on an Ortho Cytofluorograf 50H equipped with a Spectra Physics argon-ion laser producing 200

mW at 488 nm and an Ortho 2150 computer system. Forward and orthogonal light scatter, the peak and area of the red fluorescence were all recorded. After gating on a cytogram of peak versus area of the red fluorescence, to remove debris and clumped nuclei from the analysis [24], a cytogram of orthogonal versus forward light scatter was displayed on the monitor. By gating on light scatter, separate DNA histograms of the tumour and normal cells were produced. The histograms were transferred to an IBM compatible PC; further analysis and production of diagrams was performed using software written by one of the authors (MGO).

Results

An analysis of the results in the samples from palpable breast tumours which have undergone immunostaining for ER, PgR and c-*erb*B-2 has shown identical results in 19/20 (95%) cytospin preparations with a cellularity scored as C5/+++ compared to tissue sections taken from the same tumour after surgical excision (see Fig. 1, 2). There was one exception which occurred in a tumour with a predominant intraduct component which gave a false positive result for c-*erb*B-2 on the cytospin sample. This case has been reported by us elsewhere [12]. However in the series of 26 samples with between 50–400 cells/slide (++ score) only 7 cases (27%) had sufficient cells per slide for immunocytochemical detection of these epitopes. The remaining slides were considered uninterpretable due to the small numbers of cells on each slide.

In order to improve the efficiency of FNA, two consecutive samples were taken from the same tumour with a 'wet' needle followed 1 week later by a repeat aspirate using a dry needle. The results of this in a series of 32 patients are shown in Table 4 and show no significant difference in the two methods when analysed by a Mann Whitney test. The results also suggest that no significant alteration in cellularity is seen for samples taken 1 week apart from the same tumour by the same aspirator.

However there was a significant difference ($P<0.005$ using a Mann Whitney test) in the result for cytospin samples taken immediately following a standard FNA (Group 1), compared to one taken either at first presentation or following a one week gap after a standard FNA (Group 2): an examination of the results in Tables 5 and 6 shows that only 41/83 (48%) of cytospin samples in group 1 were scored as C5/+++ compared to 94/123 (75%) in group 2. This improvement in efficiency was

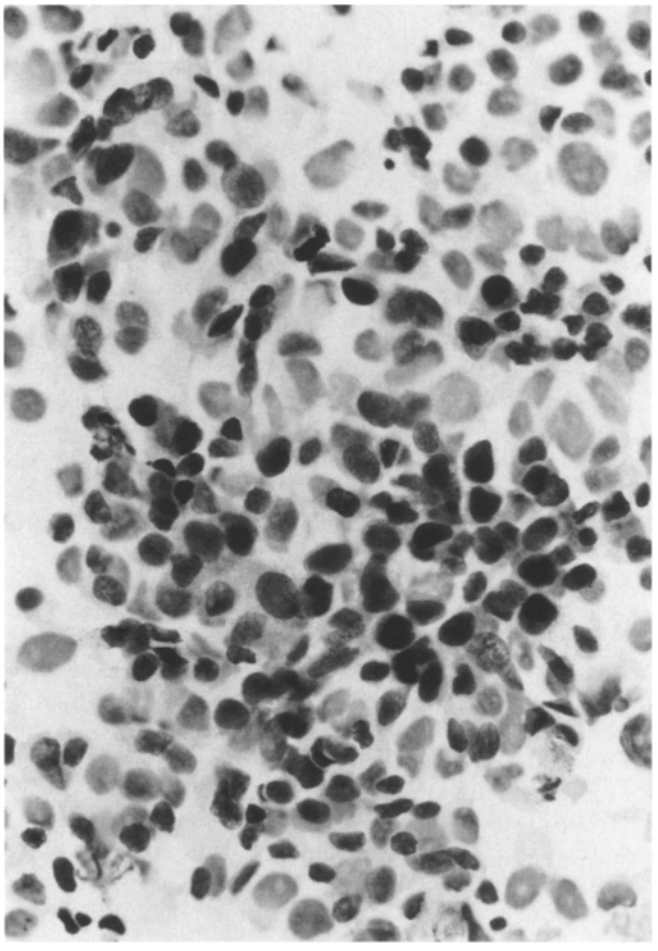

Fig. 1 Positive immunostaining for oestrogen receptor as seen on a frozen cytospin preparation, magnification ×500

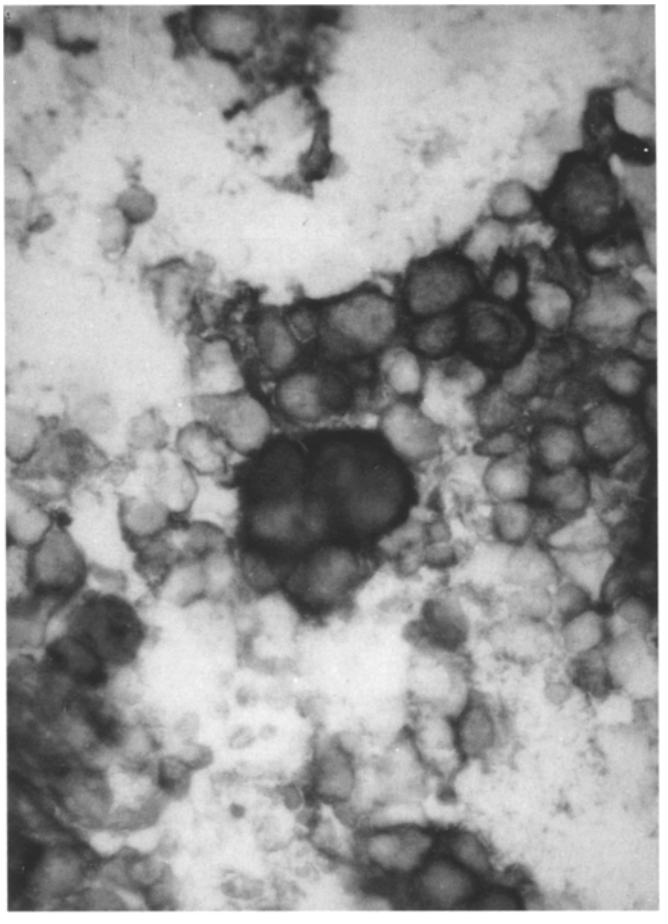

Fig. 2 C-*erb*B-2 gene product expression as detected on a cytospin preparation. Note the predominant membrane staining, magnification ×500

Table 4 The cellularity of FNAs, prepared by the cytospin technique, comparing an FNA taken with a wet needle (pre-filled with 0.2 ml of tissue culture medium) and syringe to one with a dry needle and syringe taken one week later from the same tumour

Cellularity	Dry needle			
Wet needle	C5/+++	C5/++	C5-2/+	Total
C5/+++	11	1	2	14
C5/++	2	3	1	6
C5-2/+	1	1	10	12
Total	14	5	13	32

Table 5 (Group 1) Comparison of the cellularity between a standard FNA and an aspirate prepared by cytocentrifugation with the latter taken from the same tumour immediately following the standard FNA

FNA Score when taken as 1st aspirate	Cytospin score (taken as 2nd aspirate immediately following standard FNA)				
	C5/+++	C5/++	C3-5/+	C2/+	Total
C5/+++	40	18	6	0	64
C5/++	1	1	6	0	8
C3-5/+	0	1	3	0	4
C2/+	0	0	0	7	7
Total	41	20	15	7	83

Table 6 (Group 2) Comparison of the cellularity between a standard FNA and an aspirate prepared by cytocentrifugation with the latter taken first or with at least a one week gap between the two aspirates

FNA Score when taken as 2nd aspirate	Cytospin score (with aspirate for cytocentrifugation taken before standard FNA or at least one week after standard FNA)				
	C5/+++	C5/++	C3-5/+	C2/+	Total
C5/+++	89	9	9	0	107
C5/++	3	3	0	0	6
C3-5/+	2	0	5	0	7
C2/+	0	0	0	3	3
Total	94	12	14	3	123

not seen in the results for standard FNAs and a further analysis using a Chi-squared test for partial independence has confirmed that this improvement in the cellularity of the cytospin samples was highly significant ($P<0.005$) and independent of any improvement in general sampling technique which would have occurred in all FNAs irrespective of whether they had been taken as a standard FNA or cytospin.

The distribution of cellularity of the cytospin samples in relation to the T-stage of each tumour sampled is shown in Table 7A. The results show that 114/172 (66%) of T1 or T2 tumours achieved cytospin samples of (+++) cellularity. This compared to 13/26 (50%) of T3/T4 tumours and was not statistically significant (Fisher's exact text $P<0.1$). In all eight cases where the aspirate had been taken from a palpable lymph node, cellular cytospin

Table 7A,B Distribution of cellularity of cytospin samples in relation to T-stage of the tumour in all pre-treatment samples (7A) and those in group 2 (7B)

7A	(Cellularity)			
Stage	C5/+++	C5/++	C5-C2/+	Total
T1/T2	114 (66%)	25 (14%)	33 (20%)	172 (84%)
T3/T4	13 (50%)	7 (27%)	6 (23%)	26 (12%)
Lymph node	8 (100%)	0	0	8 (4%)
Total	135	32	39	206

7B	(Cellularity)			
Stage	C5/+++	C5/++	C5-C2/+	Total
T1/T2	76 (77%)	8 (8%)	15 (15%)	99 (84%)
T3/T4	10 (63%)	4 (25%)	2 (12%)	16 (12%)
Lymph node	8 (100%)	0	0	8 (4%)
Total	94	12	17	123

Table 8 Measurement of DI and SPF as dependent on cellularity of FNAs from breast tumours in study (*DI+* or *SPF+*, DNA index or SPF measurable flow cytometry; *DI–* or *SPF–*, DNA index of SPF not measurable by flow cytometry)

	Flow cytometry				
Cellularity	DI+ SPF+	DI+ SPF–	DI– SPF+	DI– SPF–	Total
C5 ++ or ++	88	19	3	17	127
C5-C2 +	19	5	0	13	37
Total	107	24	3	30	164

aspirates were obtained in every case. A further subanalysis of patients in Group 2 divided by T-stage (see Table 7B) still confirms that 76/94 (80%) of T1/T2 tumours were scored as C5/+++ compared to 10/16 (62%) for patients with T3/T4 disease. Although this was not statistically significant (Fisher's exact test $P<0.1$) the number of patients in the latter group is too small to make any definite conclusions on the effect of T stage.

Histological classification of the breast tumours was possible in 148/203 cases. The remaining cases were treated with primary medical therapy and could not be assessed in terms of histological subtype. 137/148 (92%) were of ductal origin. 7/148 (5%) were lobular, two cases were medullary and there was one case each of mucoid and tubular type. Sixty-five percent (90/137) of all ductal tumours were scored as C5/+++ while only 1/7 (14%) of lobular type scored the same result. This result was significant (Fisher's exact test $P<0.01$) even taking into account that 2/7 lobular tumours had been included in group 1 and had been sampled by the original method.

In only 10/206 (5%) cases were both FNA and cytospin samples scored as only C2/+ when the lesion was

Table 9 Causes of failure to obtain proliferation factors as shown in Table 8

Cellularity	Cause of failure to record either DI or SPF		
	Inadequate cellularity or degraded	Overlapping peaks on DNA histogram	Total
C5 +++/++	18	21	39
C5-C2 +	16	2	18
total	34	23	57

eventually found to be malignant. For samples taken in group 2 there were only 3 such cases (2%). There were however 14 cytospin samples in the latter group, scored C3-5/+, which were suspicious of malignancy but were too poorly cellular to give a definite diagnosis of malignancy.

A total of 164 samples were analysed by flow cytometry to determine both DI and SPF. The results are shown in Table 8 and indicate that providing the cellularity on a cytospin taken from the same aspirate has been scored either +++ or ++ then the DI and SPF was detectable in 107/127 (85%) and 91/127 (71%) of cases respectively. For samples scored as only + cellularity, these same measures of proliferative index were recorded in only 24/37 (64%) and 19/37 (51%) of aspirates respectively. This difference is statistically significant ($P<0.003$ Chi-squared test).

The causes for failure to obtain either DI or SPF for the different groups is shown in Table 9. The results indicate that in 16/18 cases in the low cellularity group (+) the main cause was either sample degradation or insufficient cells for analysis. In the +++/++ group inadequate cellularity accounted for only 18/39 (46%) of cases. In the remaining 21 samples in the latter group other factors such as overlapping or multiple peaks on the flow histogram made measurement of either DI and or SPF impossible.

Discussion

The use of cytocentrifugation to obtain a cytological diagnosis from body fluids such as a pleural or ascitic fluid [5] as well as from palpable breast tumours has been described previously [8, 9] although the results have previously been disappointing in terms of efficiency with one study obtaining cytodiagnosis in only 50% of cases [9].

The present study has attempted to improve on this result as well as investigating the ability to determine a range of biochemical and proliferative factors predictive of both response to therapy and prognosis. This has been assessed using immunocytochemical staining methods and flow cytometry, on material contained within a single FNA from a breast tumour.

Our results confirm that having sufficient numbers of cells on each slide is a major pre-requisite for the determination of these immunocytochemical epitopes but nevertheless, using this technique of cytocentrifugation, it is possible to obtain 10–12 slides suitable for both cytodiagnosis as well as the detection of ER, PgR, and C-*erb*B-2 by immunocytochemical methods in over 70% of breast aspirates from palpable tumours which are considered to be suspicious of malignancy on clinical and mammographic criteria.

The technique of cytocentrifugation has several advantages over standard FNA. Firstly the cells are concentrated and deposited over a small area, reducing the quantity of antibody sera (both primary and secondary antibody) required for immunocytochemical staining. This can produce considerable savings in terms of cost of materials. Secondly the slides are of equal quality, making assessment of different stains easier and more comparable. Thirdly we have shown the ability to make more slides (10–12 slides) for other immunocytochemical stains [12] than is usually possible from a routine FNA (4–5 slides). Finally our data has shown that it is possible to obtain and adequate sample for cytocentrifugation even after a standard FNA has been taken providing a gap of 1 week has been left between the aspirates.

There are also several advantages in using a FNA over a trucut biopsy, particularly in the context of a study of primary medical therapy in breast cancer where the required information (for diagnostic or biological studies) must be obtained with the tumour remaining in-vivo. Firstly FNA produces far less trauma to the patient without any need for local anaesthesia or skin incision. Furthermore in our experience, patients who have a trucut biopsy have a much greater degree of haematoma formation in comparison to FNA [26, 34]. The subsequent swelling and oedema resulting from this makes assessment of the tumour difficult when recording the response in these patients to primary medical therapy. A greater degree of haematoma formation may also result in a worse cosmetic result for the patient after surgical excision and breast irradiation [36].

There is also no evidence that survival rates are in anyway different for patients undergoing fine needle aspiration compared to those treated by primary excision [2, 10, 27]. No such data exist for patients undergoing trucut biopsy and therefore the increased trauma associated with a trucut biopsy may result in greater vascular dissemination of malignant cells [28].

A further advantage of FNA over trucut biopsy relates to the technical difficulties in performing the latter on tumours which are less than 2 cm in diameter especially if they are deep seated making access to the lesion almost impossible to a trucut needle.

These factors have to be weighed against any potential disadvantages in using FNA rather than trucut biopsy. The most important of these is the inability of FNA to distinguish an invasive ductal carcinoma from ductal carcinoma-in-situ (DCIS) [1, 35]. This problem can be significantly reduced by including only palpable lesions which are mammographically dissimilar to pure DCIS and are greater than 3.0 cm in diameter as these are far

more likely to contain areas of microinvasion [19, 30, 31]. In the present study of over 200 cases there were 4 cases of pure DCIS but all of these cases were less than 3 cm in diameter. It should also be noted that it is still possible to misdiagnose invasive carcinoma for DCIS and vice versa even on a trucut biopsy.

A second problem is that no information on tumour grade or lymphatic and vascular invasion (LVI) is obtained on needle cytology. However, these factors cannot usually be assessed by a trucut biopsy and a negative finding, especially for LVI, could be purely due to sampling. Tumour grade can also be unreliable especially in material from a trucut biopsy. Inconsistent results have been reported in terms of reproducibility in assessing tumour grade between different centres as well as individual pathologists even when the whole tumour has been examined [16, 32]. A more objective method of assessing tumour grade using flow and static cytometry has recently been reported and would also be easily applicable to cytological aspirates [14].

The other main objection to using FNA is the lack of sensitivity and specificity of FNA compared to trucut biopsy. However in the present study cytodiagnosis was possible in over 85% of cases (including both C5/+++ and C5/++ cases with no false positives. At our own institution, a study by [25] reported no false positive cases for a C5/+++ or ++ diagnosis in 868 patients with a sensitivity of 69% for patients with definite carcinoma, using only fine needle aspiration cytology. It should be noted that patients included in this study were a selected group who were thought to have a malignant lump on both clinical and mammographic grounds. Nevertheless there have been no false positives providing the cytospin has been scored C5/++ or C5/+++. It should also be born in mind that false positive cases have been reported after trucut biopsy especially when using frozen tissue sections and papillary tumours. Areas of crush artefact or sclerosing adenosis seen within a trucut biopsy can also be difficult to interpret [4, 33].

The rather poor results seen on taking FNAs from lobular tumours was unexpected, especially as the result was statistically significant. One study in the literature has also previously commented on the low yield of cells in FNAs from lobular tumours [20].

It was possible to measure both DI and SPF in 131 (80%) and 110 (67%) cases respectively. The results showed a significant difference in those samples scored in terms of cellularity as +++ or ++ compared to +. The flow cytometer needs to analyse a sufficient number of cells (>5000) in order to determine both DI and SPF accurately especially as any DNA histogram with a coefficient of variation of >10% is usually excluded from analysis [24]. However in addition to having sufficient cells for analysis, other problems may be present in the DNA histogram such as overlapping of either an aneuploid and diploid or multiple aneuploid peaks. These can in particular prevent the measurement of SPF. This is a well recognised phenomenon which has previously been described [18].

We conclude in this study that it is possible to determine both cytodiagnosis as well as a range of immunocytochemical and flow cytometric prognostic factors with a single FNA from a breast tumour, using the method of cytocentrifugation. It is important to obtain the cytological aspirate preferably at immediate presentation or if this is not feasible a gap of one week between aspirates does not seem to alter the efficiency of the technique even when two cytospin samples are taken from the same tumour. No aspirate should be taken immediately following a standard FNA as this results in a poorer outcome in terms of cellularity. Other factors such as T stage or pre-filling the needle with fluid did not seem to make any significant difference to the cellularity of the aspirates but these latter results are based on the analysis of small numbers of patients and must therefore be viewed with caution.

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