PREOPERATIVE FINE NEEDLE ASPIRATION FROM HUMAN BREAST CANCER IS A VALUABLE SAMPLING MATERIAL FOR PROGESTERONE RECEPTOR AND CYTOMETRIC DNA ANALYSIS

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In a breast cancer series ($n = 54$), preoperative fine needle aspiration (FNA) was compared with biopsy at primary surgery as a source of material for the determination of progesterone receptor (PgR) content by enzyme immuno assay. The respective results manifested a strong correlation ($r_s = 0.82$). The fact that PgR content was usually higher in FNA samples than in the corresponding biopsy samples and the finding that 11% of the tumours were PgR positive in FNA but PgR negative in the corresponding biopsy samples suggest a greater proportion of malignant cells to be obtained with FNA than in surgical biopsy. In another breast cancer series ($n = 50$), corresponding comparisons for DNA flow cytometry showed concordance in ploidy status (diploid vs. non-diploid) in 84% of cases and a strong correlation in S-phase fraction values ($r_s = 0.70$). At DNA image cytometry, concordant results (Auer I + II vs. Auer III + IV) were obtained in 87% of the cases. To sum up, FNA seems to be a useful sampling technique for PgR determination and DNA cytometry.

Preoperative fine needle aspiration (FNA) is a valuable technique frequently used for the diagnosis of breast lesions before the final histological examination has been made. A number of studies have been reported in which a variety of approaches and variables have been used to obtain additional biological information in conjunction with FNA of the breast—e.g. steroid receptor status (1–3), flow cytometric (FCM) DNA analysis (3–9), image cytometric (ICM) DNA analysis (10), ^{3}H-thymidine incorporation (11), bromodeoxyuridine (12), Ki 67, p53 (12, 13), and morphological aspects (5, 14, 15). As an alternative to FNA for FCM DNA analysis, material was also obtained by cytopuncture in vivo (16, 17).

The clinical rationale for analysis of prognostic factors in preoperative specimens is the possible use of neoadjuvant therapy as primary medical treatment of breast cancer, including chemotherapy and/or tamoxifen. Moreover, the measurements of biological factors in FNA may also be useful in inoperable breast cancer, metastatic lesions and for monitoring treatment effects.

Since most of our knowledge concerning the prognostic and treatment predictive value of biological factors in breast cancer is based on analysis of biopsy samples taken at primary surgery, it is necessary to correlate values obtained with the two sampling materials before the usefulness of FNA as sampling material can be established. In some of the above-mentioned studies where such correlations between biopsy and cytological specimen values have been analysed—e.g. ER and PgR (1–3), FCM DNA analysis (3, 16, 17), the general conclusion has been that reliable results can be obtained by FNA, provided sufficient cellularity is available.

The S-phase fraction (SPF) as measured by FCM is one of the factors currently used clinically for prognostic purposes in breast cancer patients. The view of the Consensus Conference in 1992 was that SPF is of practical use in breast cancer, but clinical decisions must take multiple considerations into account and cannot be based solely on the SPF (18). In our Health Care Region, a prognostic index based on SPF (in some cases replaced by the Auer

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classification obtained with ICM), PgR and tumour size has been found useful for prognostic purposes among patients with lymph node negative breast cancer (19). To our knowledge, only one investigation has focused on the correlation between SPF analysis in FNA and biopsy, a study manifesting comparable SPF values (3).

The aim of the present study was to compare results of FCM SPF and DNA ploidy status, ICM Auer classification, and PgR in FNAs with the corresponding results in subsequent surgical biopsies.

Material and Methods

Study design

Two different series were used for the comparison of results obtained in preoperative FNAs and those obtained in corresponding biopsies taken at the primary operation.

Series I: PgR (n = 54). FNAs for PgR measurements were obtained preoperatively with 0.7 mm (outer diameter) needles in connection with cytological diagnosis of breast cancer (20). The aspirates were injected into 0.5 ml chilled ETN-MTG buffer (1.5 mM EDTA, 10 mM Tris, 5.0 mM Na_2MoO_4, 1.0 mM monothioglycerol, pH 7.4 at +20°C). In a parallel aspirate, the presence of cancer cells was verified cytologically. Only samples containing cancer cells were included in the present series. Cellularity was determined by counting nuclei in a Bürker chamber. Remaining samples were, within one h from aspiration, frozen at −80°C until analysed (within two weeks). At the time of analysis the samples were thawed and centrifuged at 20 000 g for 10 min. The supernatant was used for PgR analysis (see below), and the pellet for DNA determination with a colorimetric method (21) after extraction with trichloroacetic acid (22). In our study concerning ER, DNA was found to be a better reference variable than cytosol protein for expressing receptor content (1).

Tumour tissue obtained at primary surgery was sectioned and frozen at −80°C until analysed (within two weeks). At the time of analysis, the tissue was pulverized and suspended in 20 volumes of ETN-MTG buffer. Aliquots for DNA determination (see above) were withdrawn, and the remaining homogenate was centrifuged at 20 000 g for 10 min. The supernatant was used for measurement of PgR (see below). The representativeness of the samples was confirmed by the presence of cancer cells on the imprint from the biopsy.

Series II: Flow cytometric and image cytometric DNA analysis (n = 50). FNA was obtained as for PgR. Material for FCM DNA analysis was obtained preoperatively in connection with the cytological diagnosis of breast cancer. Presence of cancer cells was verified in all samples. The aspirates were injected into 1 ml citrate buffer (250 mM sucrose, 40 mM trisodium citrate 40, 5% dimethyl sulphoxide, pH 7.6). Remaining samples were frozen within one hour after aspiration and kept at −80°C until analysed. At the time of analysis the samples were thawed and centrifuged at 400 g for 10 min. The pellet was suspended in a nuclear isolation medium containing propidium iodide (PI) (50 μg PI/ml, 0.1 mg/ml RNAse and 0.6% (v/v) Nonidet P40 in isotonic phosphate buffered saline). Chicken (CRBC) and trout red blood cells (TRBC) were used as internal controls (23).

One piece of tumour tissue adjacent to that used for PgR (and ER) analysis was taken for FCM DNA analysis as previously described by Baldetorp (24). Presence of cancer cells was verified by examination of imprint. In brief, 50–100 mg tumour tissue was thawed in citrate buffer containing CRBC and TRBC, i.e. approximately 10^6 nuclei per ml (23). To increase cell elution, the tissue was mechanically disintegrated with two forceps, after which 1–2 ml nuclear isolation medium containing PI was added.

Analytical methods

Enzyme immuno assay (EIA) of PgR was performed according to the kit instructions (Abbott Laboratories, Diagnostic Division, North Chicago Ill., USA). PgR concentrations were expressed in fmol PgR per mg DNA. PgR values ⩾100 fmol/mg DNA were classified as positive and those below as negative in both FNA and biopsy.

Flow cytometric DNA analysis. The samples were analysed in an Ortho 50 H instrument (Westwood MA.) or in an Ortho Cytorone Absolute (Rariton, NJ.). For each case, both FNA and biopsy were analysed with the same instrument.

Ploidy status. In accordance with the Convention of Nomenclature for DNA Cytometry (25) ploidy status was defined as follows: one DNA stemline = diploid, two or more DNA stemlines = non-diploid.

The DNA index (DI) for the non-diploid stemline was defined as the ratio of the modal DNA, i.e. the mean DNA value of G0/G1 nuclei in a non-diploid cell population in relation to the modal DNA value of the diploid cell population. The DI for the diploid cell population was set to 1.00.

The percentage of cells in the S-phase fraction (SPF) was calculated planimetrically with no background correction, assuming the S-phase compartment to constitute a rectangular distribution between the modal values of G0/G1 and G2 peaks (26). In the event of bimodality in the 2C region or a DI for the non-diploid cell population below approximately 1.3, a mean SPF was calculated. SPF was calculated exclusively in the non-diploid cell population if the corresponding G2 peaks were distinctly separated (when DI exceeded approximately 1.3). SPF was calculated in the most prominent non-diploid stem line in cases with two or more non-diploid peaks. SPF was not calculated when background debris predominated in the SPF regions of the

histogram, when the corresponding G2 peak in the histogram could not be identified or when the non-diploid stem line was small (G0/G1 < 10% of the total number of observations).

Percentage of non-diploid G0/G1 nuclei. In order to evaluate any difference in the size of the non-diploid G0/G1 peak in biopsies and FNAs, the following formula was used: the percentage of non-diploid G0/G1 nuclei = Σ non-diploid G0/G1 nuclei × 100/Σ all G0/G1 nuclei.

As an assessment of the quality control of the DNA analysis, the full peak CV (coefficient of variation) was calculated for the diploid G0/G1 peak, as previously described (27). Debris, considered to represent PI-stained DNA fragments from destroyed nuclei, was estimated as the percentage of events appearing below the first G0/G1 peak in the DNA histograms (with the exception of those contributed by CRBC and TRBC).

Image cytometric DNA analysis

FNA. In brief, the diagnostic slides were destained in a multistep procedure with xylene, absolute ethanol (2 min), 95% ethanol (2 min), 70% ethanol (2 min), the same volume of 70% ethanol and 2 M HCl (until the colour dissappeared), 70% ethanol (2 min) and distilled water. The slides were then air-dried and fixed in 4% neutral buffered formalin and Feulgen stained as described previously (28, 29). Integral optical densitometric assessment of the nuclear DNA content was performed by means of an image analysis system purchased from Innovative Vision AB (LabEye 3PC). For each specimen, the integral optical density of 100 cytodiagnostically identified, randomly selected and well-preserved cell nuclei was determined at a wavelength of 540 nm. Internal granular leukocytes were used as 2C reference cells for ploidy assessment and as a control for the Feulgen staining. The linearity in the staining and instrumentation was checked by using Feulgen stained rat liver cells. Linearity was obtained at least up to a nuclear DNA content corresponding to 8C.

Tumour tissue. Imprints of the surface from the biopsy used for FCM DNA analysis were air-dried. At analysis, the imprint was fixed in 4% neutral-buffered formalin for at least 30 min and Feulgen stained as described above. Imprints of nuclei from human cerebellum (fresh autopsy material) on the same slide as the sample were used as 2C external reference cells.

The interpretation of the ICM histograms was performed according to the Auer classification (I–IV; 30). In this system Auer I corresponds to diploid/near-diploid with a low proliferation rate, Auer II to tetraploid with a proliferative activity of less than 5%, Auer III to diploid/near-diploid with a proliferation rate ⩾5% or aneuploid (hypotetraploid (<4C)) with a low (<5% above 4C) proliferation rate, and Auer IV to hypotetraploid or tetraploid with a high proliferation rate (⩾5% above 4C) or hypertetraploid component consisting of at least 5% of the events in the histogram.

Statistics. Subgroup differences were assessed with Pearson's χ^2 analysis. Correlations were calculated according to one-sided Spearman's rank correlation (r_s), p-values less than 0.05 being considered statistically significant.

Results

PgR

In series I the PgR concentrations in FNA manifested a strong correlation with the corresponding values in biopsy (Fig. 1; $r_s = 0.82$; $p < 0.001$). As can also be seen in Fig. 1, higher values were obtained in FNA than in the corresponding biopsy in 83% (45/54) of cases. The median quotient of FNA/biopsy values for the PgR concentration, calculated for those samples with values above method sensitivity, was 2.8 ± 10 (range 0.07 to 56). Concordance in terms of PgR positivity and negativity between FNA and surgical biopsy was obtained in 87% (47/54) of cases (Table 1). Of the seven discordant cases, PgR positivity in FNA and negativity in biopsy were obtained in six cases, and PgR negativity in FNA but positivity in biopsy in only one case.

Flow cytometric DNA analysis

In series II the CV value in frozen samples was 3.6 ± 0.9% (median 3.3%; 2.4–7.5%) and in FNA the corresponding figures were 4.3 ± 1.3% (median 4.2%;

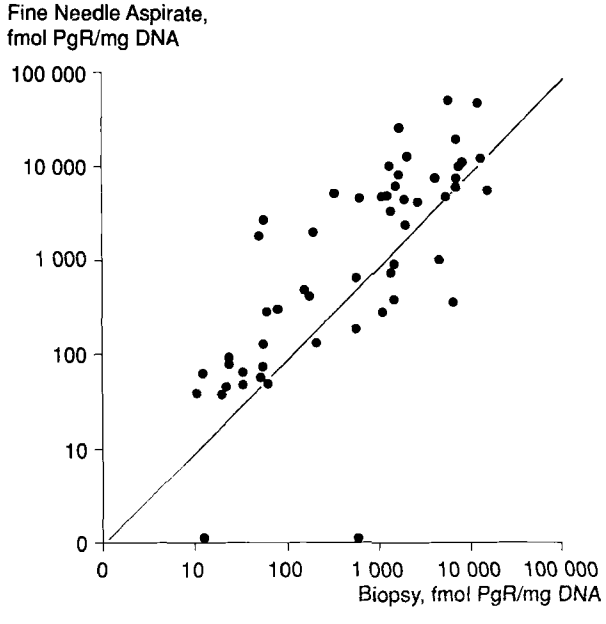

Fig. 1. Comparison of PgR concentration in fine needle aspirates and biopsies in 54 cases. The 45° slope is indicated.

Table 1

Results for PgR status (PgR −/PgR +) compared in surgical biopsy and FNA material from the same breast cancer patients

		FNA	
		PgR −	PgR +
Biopsy	PgR −	12	6
	PgR +	1	35

range 2.4–7.9%). In 84% (42/50) of cases, the same ploidy status was obtained (14 diploid and 28 non-diploid). Of the discordant cases, 4 were diploid in FNA but non-diploid in biopsy, one being near-diploid (DI = 0.96), and three having DIs of 1.88, 1.63 and 1.71. The CV for the sample diploid in FNA but near-diploid (DI = 0.96) in biopsy was 4.0%, and thus not higher than normal. The sizes of the non-diploid peak for the three remaining latter samples were small, 2.1%, 2.7% and 9.5%, respectively.

In the other group of discordant cases (i.e. diploid in biopsy but non-diploid in FNA), one was near-diploid (DI = 0.95 in FNA) with a CV of 4.1% in the corresponding biopsy sample, and three had non-diploid cell populations in FNA with DIs of 1.80, 1.92 and 2.00. The sizes of the non-diploid peak for the three latter samples were 4.1, 24 and 48%, respectively.

Regarding the number of cell populations of the 28 non-diploid cases both in FNA and in biopsy samples, 22 had two cell populations in both sampling materials, four cases had two cell populations in the biopsy but three in FNA, whereas two cases had three cell populations in the biopsy sample but two in FNA.

The DNA indices for the 50 cases yielded an r_s-value of 0.76 (Fig. 2). With one exception divergent DIs were found in cases also differing in the number of cell populations between FNA and biopsy. In 19 of the 26 cases where a non-diploid peak was found in both FNA and frozen biopsy, the size of the non-diploid G0/G1 peak was calculated. The mean percentage of non-diploid G0/G1 nuclei was found to be higher in FNA than in the corresponding biopsy (52 ± 23% vs. 37 ± 19%; Fig. 2).

The S-phase fraction (SPF) was estimated in 90% (45/50) of biopsy samples and in 88% (44/50) of FNA samples. The SPF was 8.7 ± 5.7% (median 6.9%) in biopsy samples, and 9.1 ± 6.1 (median 8.0%) in FNA. Subgrouped with regard to ploidy status, the mean SPF for diploid cases was 5.1 ± 2.9% (median 4.1%) for biopsy samples and 4.5 ± 2.8% (median 3.7%) for FNA samples. The corresponding figures for the non-diploid subgroup were 11 ± 5.8% (median 9.6%) and 12 ± 5.7% (median 11%). The correlation of SPF between frozen biopsy samples and FNA is shown in Fig. 2, yielding an r_s-value of 0.70. The

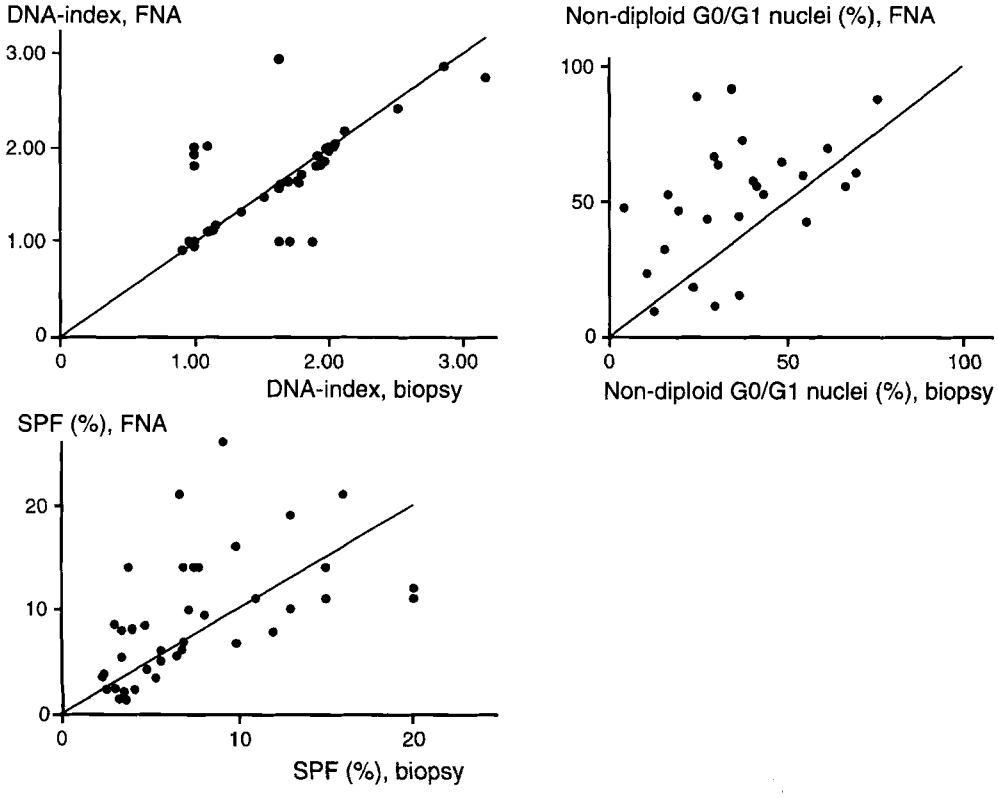

Fig. 2. Comparison between fine needle aspirates and biopsies from breast cancers for FCM analysis of DNA-index (n = 50), the percentage of non-diploid G0/G1 nuclei (n = 26), and FCM S-phase fraction (n = 41). The 45° slope is indicated.

Table 2

Results in terms of Auer classes (I–IV) compared in surgical biopsy and FNA material from the same breast cancer patients

		FNA			
		I	II	III	IV
Biopsy	I	10	3		
	II	5	1	2	2
	III		1		3
	IV	1			18

r_s-value was 0.48 for the diploid subgroup (i.e., diploid in both sample materials) and 0.52 for the non-diploid subgroup.

Image cytometric DNA analysis

In series II, one histogram of frozen biopsies and three histograms from FNA were considered non-evaluable. The mean full peak CV was 5.6 ± 2.5 in biopsy material and 7.7 ± 2.5 in FNA. Of the 46 cases evaluable in both sampling materials, a complete concordance in Auer classes was obtained in 63% (29/46) of cases (Table 2). For prognostic purposes Auer I and II are considered to represent a low risk of recurrence, whereas Auer III and IV represent a high risk of recurrence (10, 30). Using these subgroups instead, concordance increased to 87% (40/46). Four of the discordant cases were Auer II in biopsy samples but either Auer III ($n = 2$) or Auer IV ($n = 2$) in FNA. One case was Auer II in the FNA but Auer III in the biopsy sample.

Comparison of FCM and ICM results

In a previous investigation by our group, approximately 20% of DNA diploid samples manifested an abnormal DNA content (Auer II-IV) at ICM (31). In the present series, 33% (6/18) of cases in the biopsy group were diploid at FCM but Auer II–IV at ICM, as compared with only 18% (3/17) of cases in the FNA group.

Discussion

In the present study, EIA values for PgR content obtained in FNA samples were found to manifest a strong correlation with those obtained in corresponding surgical biopsy samples. A similar strong correlation was obtained for ER values in a previous study in our laboratory (1), and also by other groups (2, 3). Moreover, as previously found for ER content, the PgR content was usually higher in FNA than in biopsy samples. This can probably be explained by the greater proportion of malignant cells obtained by FNA, whereas in biopsy samples the amount of non-cancerous cells present (e.g. connective tissue fibroblasts or inflammatory cells) may reduce the value obtained for receptor content, as expressed per mg DNA of the total sample. The receptor values obtained in FNA may thus be closer to the true value. Spyratos and co-workers have also suggested that the cellular material obtained in cytopunctures appears to be highly representative of the cellular component of the tumour (17). This advantage of FNA over biopsy analysis is also apparent in the present study. Of the seven discordant cases, only one was PgR negative in the FNA sample but PgR positive in the biopsy sample, whereas six were PgR positive in FNA samples but PgR negative in biopsy samples. In our previous study, where EIA values for ER content in FNA and biopsy material were compared, 18 out of 65 preoperatively taken FNAs were considered to be of low cellularity ($< 50\,000$ cells/ml). In the present study, only one (PgR negative in both FNA and surgical biopsy) was classified as cell-poor.

Both FCM and ICM analysis yielded good concordance between FNA and biopsy materials with an agreement in ploidy status in 84% of cases at FCM, and in Auer classification in 87% of cases at ICM, and a correlation ($r_s = 0.70$) in SPF values at FCM. Previous studies by other groups have shown a concordance in ploidy status above 85% (3, 16, 17). One reason for discordance in ploidy results between the two sampling materials may be intra-tumour DNA heterogeneity, as in a previous series ($n = 74$) analysis of two biopsies from each tumour yielded discordant results for ploidy status in 10% of cases (32). As FNAs are representative of a greater tumour volume, tumour heterogeneity should be less of a problem than excision biopsy restricted to one small area. Another possible explanation of the discordance is that a greater proportion of malignant cells may be obtained at FNA. This suggestion is supported by the fact that non-diploid peaks were larger in FNA material than in corresponding biopsy material (52% vs. 37% at FCM, and 83% vs. 73% at ICM), and by the fact that, as compared with biopsy samples, fewer FNA samples were diploid at FCM, and manifested abnormal DNA content at ICM. Spyratos and co-workers have also found a significant increase in the number of non-diploid nuclei in the cytopuncture compared with the tissue sample (17).

Regarding the technical quality of FCM DNA histograms, there was no significant difference in the coefficient of variation for the diploid G0/G1 peak between biopsy and FNA materials (3.6% vs. 4.3%), and the median percentage of debris was also quite similar (12% vs. 15%). At ICM, DNA histogram quality was also similar in the two materials.

As mentioned above, there was a strong correlation in SPF values between biopsy and FNA materials ($r_s = 0.70$), comparable to 0.88 in the study by Marrazzo and co-workers (3). For clinical purposes, however, cut-off levels are often adopted to define low and high SPF subgroups. At our laboratory, diploid tumours with an SPF value

$\geqslant$7.0% and non-diploid tumours with an SPF value $\geqslant$12 are classified as having high SPF, and the remainder as having low SPF (33). If these cut-off levels are applied in the present series, the concordance in SPF categories between FNA and biopsy materials would only be obtained in 68% (28/41) of cases overall, in 79% (11/14) of cases of diploid tumours, and 64% (14/22) of cases of non-diploid tumours. Although part of this discordant rate can be explained by tumour heterogeneity (32), this rather high degree of discordance in results suggests that the clinical value of SPF determined in FNA should be further evaluated, preferably in a prospective breast cancer series with clinical follow-up. Moreover, the optimal cut-off levels for SPF in FNA remain to be determined. One of the inherent problems with cut-off is illustrated by the finding that 7 out of the 13 discordant cases had SPF values within 1.0% of the cut-off level, and 12 within 2.0%.

It should also be borne in mind that the good overall results obtained in FNAs do not mean that equally good results would be obtained in FNAs of smaller lesions such as lymph nodes or distant metastases, where the proportion of normal cells may be greater and thus result in poorer accuracy in analysis. Moreover, the importance of the representativeness of the FNA cannot be overemphasized.

To sum up, preoperative FNA may be a useful alternative to surgical biopsy for the purpose of determining tumour PgR content, and in some cases is a better sampling technique. FCM results for DNA ploidy status and ICM results also seem to be reliable, whereas SPF results need to be interpreted with caution until further evaluation has been performed and optimal cut-off levels have been established for the purpose of categorization.

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