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EVALUATION OF DNA-PLOIDY AND S-PHASE FRACTION ANALYSIS BY FLOW CYTOMETRY IN HUMAN BREAST CARCINOMA.

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ABSTRACT

Breast cancer is the most common form of malignancy that affects women. Tumor proliferation can be monitored by measuring DNA synthesis using flowcytometry which provides rapid and precise analysis of large numbers of cells. The aim of the present study was to evaluate DNA ploidy in breast cancer. A number of 85 patients diagnosed with mammary carcinoma and subjected to surgery and different hospital were taken into consideration. None of the patients were subjected to chemo- or radio-therapy prior to surgery. Cell cycle analyses were performed with fresh tumour sample and normal tissue surrounding the tumour. The present study investigate flowcytometric histogram revealed that 25% of the tumour samples showed 62.99% of cells in S phase that were hypodiploid, 25% of subjects with a value of 31.89% were aneuploid and 50% showed a value of 31.89% were aneuploid. Besides, 50% of the samples indicated a S phase value ranging from 9%-20% and were deemed diploid. Data were analysed by the ModFit LT3.1 software provided by Becton Dickinson. Our study indicated that DNA ploidy could be an important factor for estimating the degree of genomic instability which may be reflected by the aggressiveness of the tumor.

KEYWORDS: Breast Cancer, Flowcytometer, S-phase Fraction, DNA- ploidy.

INTRODUCTION

Flow cytometric analysis may be the main reason for its limited use in the clinical management of breast cancer(Baldetorp, *et al.*, 2003). Flowcytometric S-phase

fraction (SPF) is generally considered to be a prognostic factor in breast cancer (Bast *et al.*, 2001).

Flowcytometric analysis was performed on preserved tumour tissues using the (Vindelov *et al*., 1983). Breast cancer is the most common form of malignancy that affects women. Tumor proliferation can be monitored by measuring DNA synthesis using flow cytometry which provides rapid and precise analysis of large numbers of cells **(**Dorina Munteanu, *et al.*, 2004). The specimens were mechanically and enzymatically dissociated to obtain bare nuclei, treated with RNAase, and then stained with propidium iodide. Non tumorous tissue or peripheral lymphocytes were used as references standards. The nuclei suspensions were acquired and analyzed on Becton Dickinson flowcytometer equipped with the laser beam and internal signals from the blue photomultiplier transducer. A total of events were collected for each sample. All histograms were analyzed independently using cell fit software.

Flowytometric data were classified (Hiddemann *et al*., 1984) as follows: diploid – when only a single primary peak was present in the same location as the control.

i) **Tetraploid** – when an abnormal peak in the tetraploid range contained more than 15% of the total cells.
ii) **Aneuploid** – when an abnormal peak (other than tetraploid) separate from the diploid peak was present that could be confirmed by the addition of control cells, the histograms being further classified as hyperdiploid, hypodiploid, multidiploid or hypertetraploid depending on the location and number of abnormal peaks.
iii) **DNA index** is calculated as the ratio of the DNA content of G0/G1 cells in the abnormal population to the Go/Gi cells of a normal, diploid population.

Flowcytometry measurement of DNA is a faster method to assess the growth characteristics of the tumour. It is now possible to carry out flow cytometry analysis of paraffin embedded specimens (Damadian, 1971) comparing the DNA index and growth fraction with other well known prognostic factors, It was found that aneuploid correlation with increased tumour size, poor differentiation and the presence of metastases (Bell *et al*., 1981 and Frey, 1972). Some investigators have also reported a high aneuploidy relation ER and progesterone receptor negative tumour (Inch *et al*., 1974). Present study also focuses on the clinical utility and potential value of cell cycle analysis and DNA ploidy interpretation in the diagnosis of human tumors, the application of these techniques to cytological diagnosis, and their capability for predicting disease outcome in human neoplasia. Methods of cell cycle analysis

are considered, and the techniques of flow cytometry and image analysis are described (Jeffrey and Ross 1996). Therefore, the assessment of factors such as cell cycle and DNA ploidy may be eventually useful to determine the response to treatment and prognosis. Breast cancer is the most common form of malignancy affecting women. The incidence of breast cancer tends to increase.

Techniques for Measuring DNA Content

Techniques used to demonstrate DNA content include flow cytometry, image analysis, and laser scanning cytometry. The flow cytometer uses an optical-to-electronic coupling system and records how a cell interacts with a focused laser beam in terms of scattering of incident light and the cell's ability to emit fluorescence. The photons of light scattered and emitted by a cell following its encounter with the laser beam are separated into various wavelengths by a series of filters and mirrors. Detectors then generate electrical impulses that are converted into digital signals that then are accumulated in a frequency distribution, or histogram. DNA content is studied most commonly by staining cells with propidium iodide, a DNA-binding dye that can be excited with a standard argon laser. Combined DNA and RNA measurements can be made with the metachromatic fluorochrome acridine orange (Traganos *et al.*, 1977). A technique also has been described to simultaneously study DNA and RNA by measuring DNA staining with Hoechst 33342 and RNA staining by pyronin Y (Ross 1996). DNA analysis by flow cytometry provides fast results, permits multiparameter analysis correlating DNA content with antigen expression, and provides the sensitivity for detecting near-diploid aneuploid peaks. However, because flow cytometry requires disaggregation of the tissue sample, there is no simultaneous morphologic comparison (Koss, *et al.*, 1989). The presence of aneuploid populations might be masked by the inclusion of numerous benign, nontumor cells. Flow cytometry also has the advantage of permitting retrospective studies of paraffin-embedded tissue samples. Although the best histograms are obtained from fresh or frozen tissue samples.

Hedley and coworkers (1983) developed a technique that permitted flow cytometry to be performed on formalin-fixed, paraffin embedded tissue sections. Fixed sections are cut from tissue blocks, dewaxed in xylene, and rehydrated through alcohol solutions. Single-cell or nuclear suspensions are obtained after incubation with a 0.5% pepsin-saline solution. Cells are counted, washed, and stained with 6-diamidino-2-phenylindole (Krause and Blank, 1992). Modifications of the Hedley technique have permitted good correlations between paraffin-

embedded specimens and histograms from fresh specimens (Shapiro, 1989) although histograms obtained from paraffin-embedded tissues have wider coefficients of variation and are, therefore, less precise (Wersto, *et al.*, 1991). Techniques also have been described for performing chromosome analysis by flow cytometry (Connell, *et al.*, 1986).

METHODOLOGY

Paraffin section 30 microns embedded tissue in a glass tube was deparaffinized using xylene. Briefly, 3ml xylene was added to the tissue sections, incubated at room temperature for 3-4 min and then centrifuged for 3 min at 950g. The used xylene was decanted taking care not to discard the tissue sections. Step 2 was repeated three times. The tissue sections were rehydrated. The deparaffinized sections were incubated in 100% ethanol at room temperature for 3 min and then centrifuged for 3 min at 950g. The used ethanol was discarded by careful aspiration. The steps were repeated with decreasing concentrations of ethanol i.e., 95%, 75%, 50% and 25%. The rehydrated sections were washed once with triple distilled water. Incubated at room temperature for 3 min and then centrifuged for 3 min at 950g. The supernatant was decanted and add 2ml of pepsin (5%) to the tissue sections. The sections were incubated for 1-3 hours at 37°C in a water bath to allow tissue disaggregation. The cell suspension was vortexed and filtered using a wire mesh to remove the large tissue aggregates. After filtration, the suspension was centrifuged for 5min at 950g. The supernatant was decanted. The pellet was re-suspended in 1ml of wash buffer and incubated for 5min, at room temperature. The cells were counted using a Hemocytometer and 1-2x 10^6 cells/ nuclei were taken for staining. Vortexed for 1-2min and thereafter centrifuged at 2350g for 2min, the supernatant was decanted. The pellet was resuspended and 250µl of solution A was added. It was incubated at room temperature for 10 min. Without discarding solution A 200µl of solution B was added. It was mixed gently by tapping and incubated for 10 min at room temperature.100µl of cold solution C was added and incubated at 4°C for 20-30 mins in dark. The cells were analyzed using a flowcytometer within 3 hrs. of staining 40,000- 50,000 cells were acquired on flowcytometer (FACS Calibur). The cells were analyzed using the ModFit LT3.1 software provided by Becton Dickinson.

RESULTS

The modal peak of the histogram is the DNA Index (DI). If this index is the same as for normal control cells, it is called diploid and has a value of 1. Generally a range of 10% is acceptable diploid (0.9 < D.I. < 1.1). Cancer, that have abnormal cellular DNA content are

called hypo diploid (DI < 0.9), aneuploid (DI > 1.1) or tetraploid (DI = 2). Tetraploidy was not observed in the present study. Flowcytometry disclosed a DNA histogram of diploid DNA content (Fig. 1) and a low percentage of S-phase cell. Peak 1 represented G_1 and G_2 cells and peak 2 represented G_2 and M cell S-phase fractions (SPF) and was found to be more due to tumour aggression while G_2–M peak appeared normal. As computed from the histogram SPF value (16.52%), was found to be higher. DNA index was observed to be 1 and ploidy found to be diploid. Flowcytometric histogram (FCH) (Fig. 2) showed a DNA Index of 1.0 but percentage of cells in S phase was found to be 9.5 and the cells were found to be diploid and without much scattering of cells.

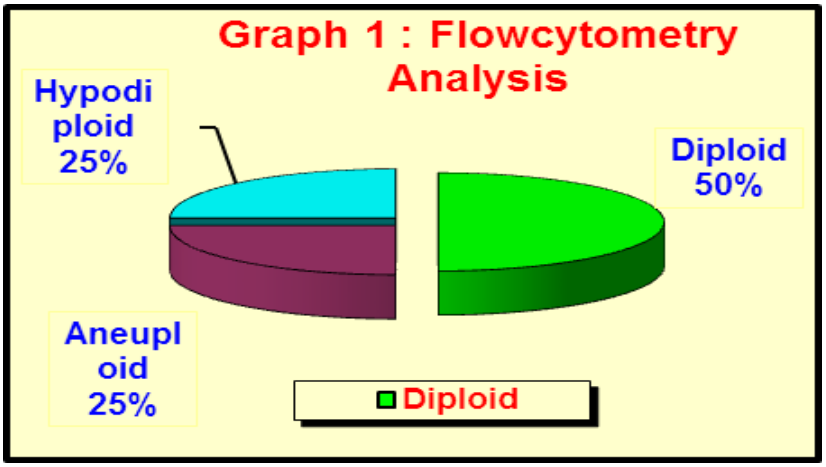
FCH showed hypodiploid breast carcinoma cells with DNA Index of 0.84 and percentage of cells in S phase was 44.31. FCH (Fig. 3) showed aneuploid breast carcinoma cells with 1.18 DI and 21.74% cells in S phase. Flowcytometry results confirmed show much scattering of cells. Fig.2 & 4 samples showed high proliferation of carcinoma cells. Flowcytometry indicated rapid multiplication of cells in the S-phase and become a more aggressive tumour with G_1–G_2 diploid peak, hypodiploid cell population with DNA index of 0.84, total aneuploid S- phase of 62.99% and total S- phase of 44.31%.

The slow pace of DNA synthesis at the beginning and end of the S-phase resulted in a disproportionate fraction of S-phase cells near the extremes of S, rather than in the middle. The presence of many S-phase cells under the G_1-G_2 peaks complicated the estimation of S-phase fraction from DNA histogram. The S-phase cells had a DNA content intermediate between those of G_1 and G_2 cells. They were distributed between the beginning of the G_1-G_0 peak and the G_2-M peak. Because of the relatively slow pace of DNA synthesis early and late in the S-phase, relatively more S-phase cells fell under G_1-G_0 and G_2–M peaks than might be expected (Fig. 3).

Among the samples that were analyzed using flowcytometry, (Table 1 & Graph 1), 50% of the samples showed a DNA Index of 1.00, which interpreted as diploidy, 25% showed a DNA Index of 0.84, being interpreted as hypodiploidy and 25% showed a DNA Index of 1.18, indicating aneuploidy. Flowcytometric histogram revealed that 25% of the tumour samples showed 62.99% of cells in S phase that were hypodiploid, 25% of subjects with a value of 31.89% were aneuploid and 50% showed a value of 31.89% were aneuploid. Besides, 50% of the samples indicated a S phase value ranging from 9%-20% and were deemed diploid.

TABLE 1: Flowcytometry Analysis

Type of Ploid	Percentage
Dipliod	50
Anupliod	25
Hypodipliod	25



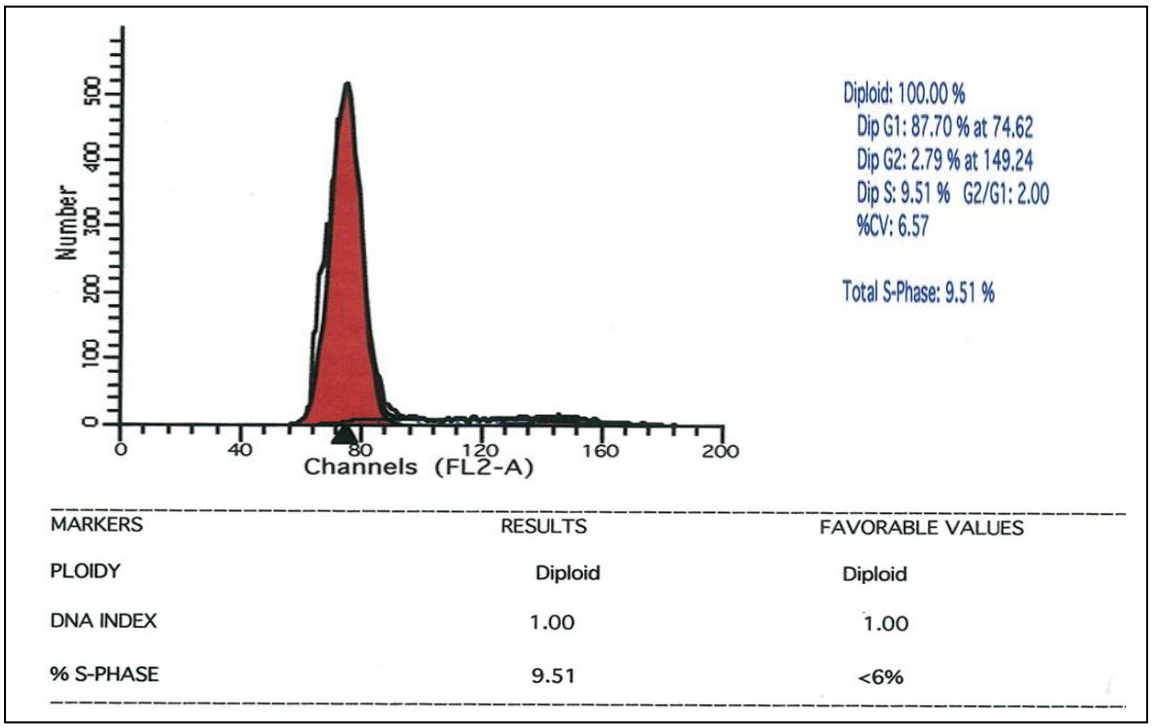
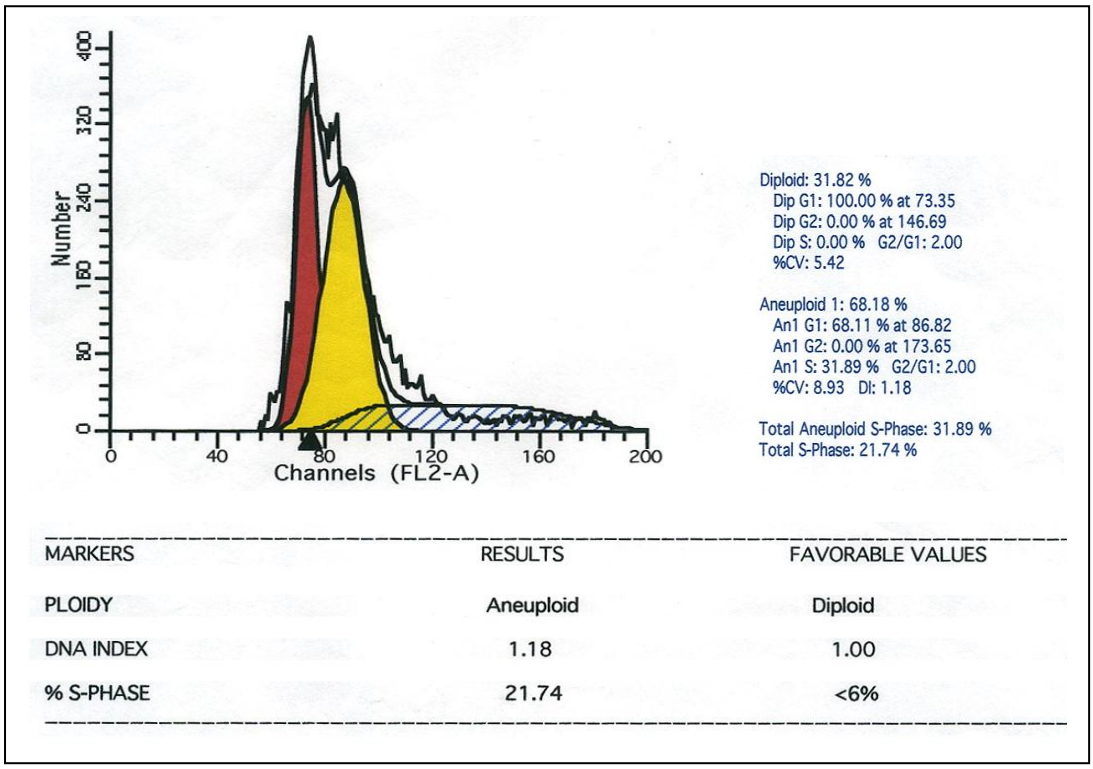
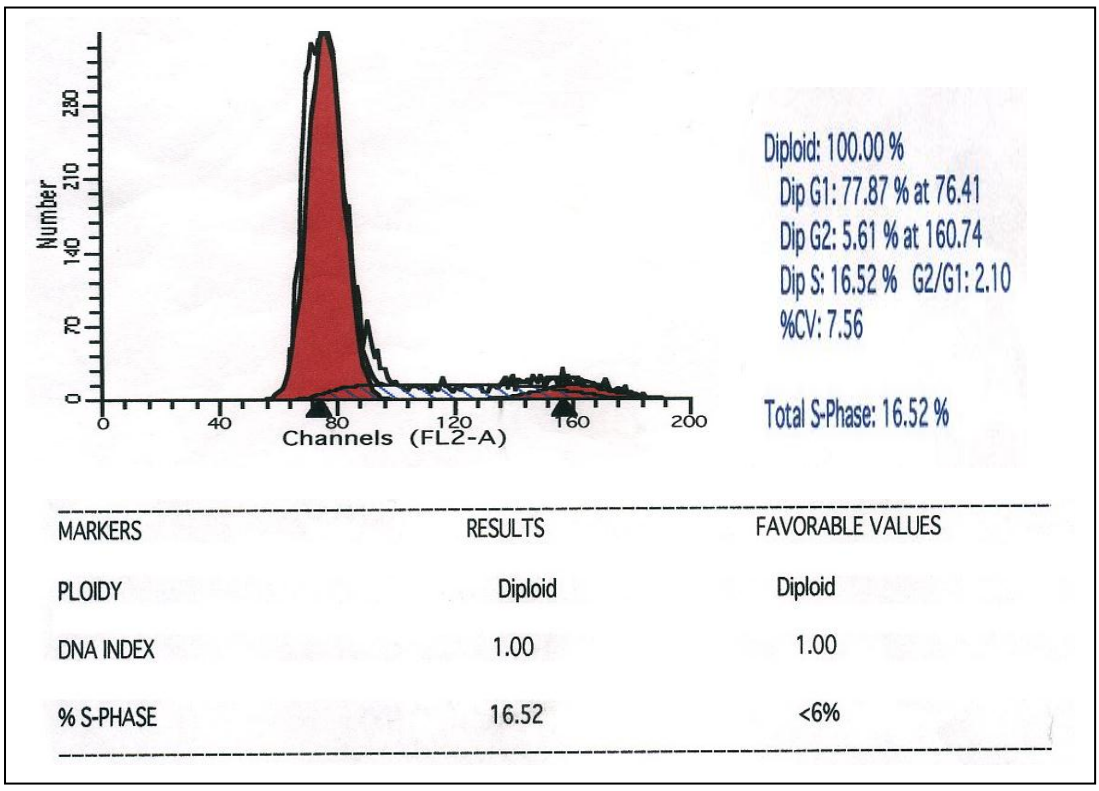


Fig. 1 Flowcytometry of Normal Breast - Control



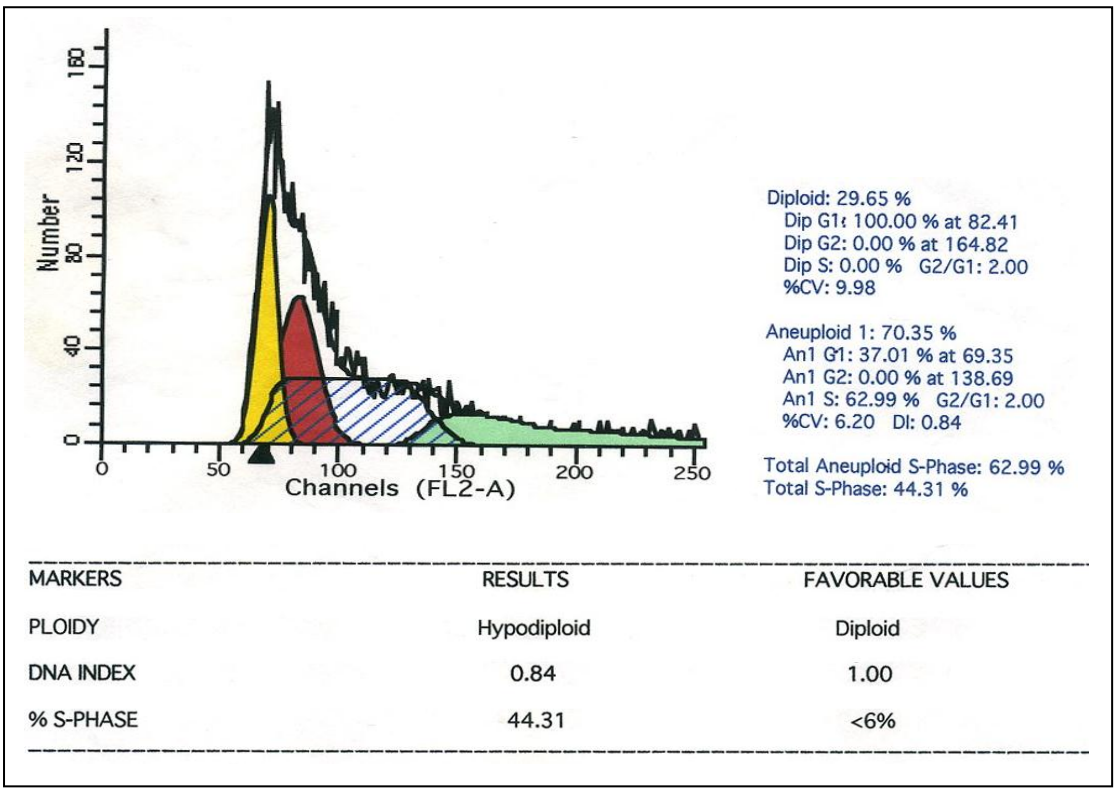
MARKERS	RESULTS	FAVORABLE VALUES
PLOIDY	Aneuploid	Diploid
DNA INDEX	1.18	1.00
% S-PHASE	21.74	<6%

Fig. 2 Flowcytometry of aneuploid cell population - DNA index and S - Phase fraction in breast carcinoma.



MARKERS	RESULTS	FAVORABLE VALUES
PLOIDY	Diploid	Diploid
DNA INDEX	1.00	1.00
% S-PHASE	16.52	<6%

Fig. 3 Flowcytometry of diploid cell population - DNA index and S - Phase fraction in breast carcinoma



MARKERS	RESULTS	FAVORABLE VALUES
PLOIDY	Hypodiploid	Diploid
DNA INDEX	0.84	1.00
% S-PHASE	44.31	<6%

Fig. 4 Flowcytometry of hypodipiod cell population - DNA index and S - Phase fraction in breast carcinoma.

DISCUSSION

Flowcytometry offers an excellent technology to correlate phenotypic characteristics of tumour cells with the malignant biological behaviour to acceptable statistical accuracy. DNA flow cytometry may be important in the prognosis of breast tumours (Suzuki *et al.*, 1997 and Bittard *et al.*, 1990). Jeffrey, *et al.*, (2003) review also considers the prognostic significance and potential clinical utility of ploidy measurements, S phase calculation, and individual cell cycle regulatory biomarker expression levels. Present study also revealed that also considers the prognostic significance and potential clinical utility of ploidy measurements, S phase calculation, and individual cell cycle regulatory biomarker expression levels.

Although aneusomy and aneuploidy often coexist, it has been reported that fluorescence in situ hybridization is more sensitive than flow cytometric– or image cytometric–derived DNA ploidy measurements for the detection of abnormal DNA content in human tumors (Waters *et al.*, 1998 and Lifson *et al.*, 1995). Equal to that of the total DNA content of the G0/G1 peak of a known-to-be-diploid reference cell population and the S and G2 M phases of the tumor cell population are relatively low. In normal tissues and most low-grade or slowly proliferating neoplasms, approximately 85% of the cell population forms the G0/G1peak and

15% of the cells are in the S and G2M phases. DNA aneuploidy, also known as nondiploidy, is defined as a DNA content of the G0/G1peak of a cell population that varies substantially from the G0/G1peak of the known diploid reference cell population **(Table)** The DNA index of an aneuploid cell population rarely might be less than 1.0 (hypodiploid) and far more commonly is greater than 1.0 (hyperdiploid). Nondiploid cell populations featuring a DNA index of the G0/G1main peak at or near 2.0 must be differentiated from diploid tumors with significantly increased G2M phases. Some investigators refer to these nondiploid tumors with DNA indices near 2.0 as tetraploid tumors. Illustrates the 4 most frequent types of DNA histograms.

By contrast with normal tissues, malignant cells are characterized by genetic instability leading to chromosomal anomalies. DNA ploidy of a cell is proportional to the fluorescence intensity of specific dyes, which have a stoichiometric interaction with DNA, and is expressed by its DNA Index. Smith *et al.,* (2003) reported that elevated DNA damage is significantly associated with breast cancer risk and define the molecular mechanism of DNA damage in breast cancer susceptibility. Ploidy analysis measures the amount of DNA contained in a cell. Most cancer cells are aneuploids, with abnormal amount of DNA. Montgomery, *et al.,* **(**1990) The DNA ploidy distribution of all pathologic stage B prostate cancers differs significantly from that found in more advanced stages (C and D1) previously reported for the same time interval. However, the ploidy distribution of stage B tumors that progressed closely resembles that of the stage C and D1 tumors. These results further support the working hypothesis that nuclear DNA content has marked prognostic significance for patients with adenocarcinoma of the prostate. It seems to us that analysis of ploidy by flow or static cytometry will become an essential tool for treating patients with localized prostate cancer.

The DNA analysis of breast cancer by flowcytometry might supplant morphological data in assessing tumor behavior (Visscher *et al.,* 1990) Aneuploid DNA content is significantly associated with markers of decreased morphological grade and biochemical differentiation. Determination of S-phase fraction by FCH appears to be rapid and potentially easy method for obtaining kinetic information on individual breast tumour. The S-phase cells have a DNA content intermediate between those of G_1 and G_2 cells. They are distributed between the beginning of the G_1-G_0 peak and the G_2-M peak. Because of the relatively slow pace of DNA synthesis early and late in the S-phase, relatively more S-phase cells fall under G_1-G_0 and G_2–

M peaks than might be expected (Culpin and Morris 1980). DNA Index is the ratio of relative DNA content of G_0-G_1, cells divided by the mode of the relative measurements of the DNA diploid G_0-G_1 reference cells. Therefore, DNA Index of a diploid cell population is 1.0 and DNA triploid or tetraploid is 1.5 or 2.0. Among the samples that were analyzed using flowcytometry, 50% of the samples showed a DNA Index of 1.0, which is interpreted as diploidy, 25% showed a DNA Index of 0.84 as hypodiploidy, 25% with a DNA Index of 1.18, as aneuploidy.

Breast carcinomas increase in size gradually and can take as long as 10 years to reach 1 cm in diameter, and as they grow larger they acquire properties that allow the cells not only to proliferate inexorably but also to spread (George *et al.,* 2001). Although in some studies of breast carcinomas, the mean SPF has been as low as 3.7% to 7.5% (Haag *et al.,* 1987 and McDivitt *et al.,* 1985). Roughly 20% to 40% of breast carcinomas has no detectable aneuploid cells and 60% have aneuploid cells. Of the aneuploid carcinomas, only about 1% are hypodiploid (less than normal DNA content), approximately 80% are hyperploid or tetraploid, and 20% are hypertetraploid (more than twice the normal DNA content).

Survival is distinctly higher for patients with predominantly diploid DNA contents (50% of the subjects under study) in the breast carcinoma cells than those with early aneuploid DNA content, (25% of the subjects under study) according to Atkin (1972), Auer *et al* (1980) and Carvey *et al* (1987). In addition aneuploid carcinomas are poor in ER, whereas diploid tetraploid carcinomas are often rich in ER (Bhargawa *et al.,* 1994). Another study showed 75% scattering of cells in DNA histograms. It also suggests that genomic instability was prominent in DNA - aneuploid tumors and supplements major stemline position as a prognostic variable (Fallenius *et al.,* 1988). Since 25% of the subjects show aneuploid breast carcinoma, they might have a higher rate of axillary metastases than DNA diploid tumors (Berryman *et al.,* 1987 and Erhardt *et al.,* 1986). They also observed increased rates of DNA aneuploidy in young women. The present study revealed that large carcinomas (25%) usually are DNA aneuploid, whereas small carcinomas usually have diploid DNA content. According to Suzuki *et al.,* (1997) the carcinoma showed diploid in the primary lesion and aneuploid during recurrence. DNA index in recurrent carcinoma was higher than that in aneuploid adenoma. Therefore, aneuploid adenoma should be strictly followed up. Zhao *et al.,* (1995) used flowcytometry to measure the DNA content in 67 cases with squamous cell carcinoma of the tongue. Michels *et al.,* 2004 reported that tetraploid tumors had approximately the

same prognosis as aneuploid tumors, whereas hypoploid tumors had a slightly better outcome than even diploid tumors. The results showed that the recurrences always appeared within 2 years after operation. 75% of recurrences were aneuploidy. The close relative factors for the cancer were identified to be clinical stage, lymph node metastases, DI value and DNA ploidy. Tumour ploidy determined by flowcytometry, is considered to be a major prognostic factor for survival in advanced ovarian cancer (Rodenburg *et al.*, 1987). The results suggested that the FCH had diagnostic value in breast carcinoma patients, especially when the morphological diagnosis was difficult, which would help the medical physician select a therapeutic method.

The vast majority of benign tumours are DNA diploid, whereas a variable percentage of malignant tumours are DNA aneuploid. Depending on their histology of DNA diploidy DNA anueploid, and DNA multiploid flowcytometric histograms have been observed (Vielh, 1995). It is also interesting to note that DNA aneuploidy has been associated with shorter overall survival in osteosarcomas (Xiang *et al.*, 1987). It was found that recurrence rate at 16 sixteen month was twice as high for aneuploidy tumour as for diploidy tumours. A prognostic test combining the DNA index and the proliferation rate was proposed (Economou *et al.*, 1973).

Three types of DNA histograms are defined, which are associated with a favorable, intermediate and poor prognosis. DNA histogram in the case of breast cancer (solid tumour) obtained at flowcytometry gives the following information,(i) the presence or absence of a DNA aneuploid stemline (ii) the relative DNA content of the aneuploid stemline, expressed as DNA index (iii) the proliferative fraction of the stemline (iv) the presence of the cell population with DNA content greater than the normal DNA diploid Go/ G1 (Herman and Walloch, 1991). Of these, DNA aneuploid has the most practical value. Fortunately there is good evidence that solid tumours are quite homogenous with respect to the presence of aneuploidy.

CONCLUSION

Breast carcinogenesis indicates that elevated DNA damage is significantly associated with the risk and defines the molecular mechanism of DNA damage repair and susceptibility. The amount of DNA contained in the nuclei of breast carcinoma cells will provide an indication of their malignant potential and flowcytometry is a means for measuring the amount of DNA. Normal cells, or those of a benign neoplasm, tend to have a single homogenous population of

cells with a "euploid" DNA content. However, malignant cells are less differentiated and have abnormal expression of DNA content. The degree of "aneuploidy" can be assured by flowcytometry. The results of our study indicate that DNA ploidy might be an important factor for estimating the degree of genomic instability which may be reflected by the aggressiveness of the tumor. Further information will be provided by analyzing a larger number of cases in order to confirm the value of DNA ploidy as a prognostic factor in the breast cancer.

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