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The Influence of Age on the DNA Ploidy Levels of Breast Tumours

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Abstract—*Primary tumour DNA content and estimates of cell cycle kinetic parameters were analysed by flow cytometry in 114 cases of breast cancer. Tumours were classified as: near-diploid, diploid, single aneuploid, tetraploid and greater, and multiploid (defined as having more than one aneuploid tumour cell population). No significant correlations were found between ploidy and histologic type, tumour size, lymph node involvement or receptor (oestrogen and progesterone) status. A highly significant correlation between ploidy and proliferative activity (as assessed by the percentage of cells in S phase) was observed, with near-diploid and diploid tumours being associated with a low (≤10%) S phase fraction (P = 0.0001). A marked relationship between ploidy and patient age was also seen, with increased DNA content being associated with older patients (P = 0.025). In contrast, no patients with multiploid tumours were over 60 yr, and their age distribution was significantly different from the population as a whole (P< 0.05), suggesting that multiploidy might be a phenomenon associated with the menopause.*

INTRODUCTION

THIS study was initiated with the long-term goal of evaluating DNA ploidy as a possible independent prognostic variable in breast cancer, with particular reference not only to long-term survival but also to the incidence of recurrence and response to subsequent therapy. Previous studies of a variety of solid tumours, including those of the breast, have demonstrated that long-term survival does correlate with DNA ploidy, with diploid tumours generally having a better prognosis [1, 2].

As an interim study the relationship between DNA ploidy levels and previously described prognostic variables [3], including histologic type, tumour size, involvement of axillary nodes, receptor status, proliferative state of the tumour and patient age, was examined here.

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MATERIALS AND METHODS

All specimens came from the Regional Biochemistry Department of Royal Prince Alfred Hospital, Sydney, where they had been sent for receptor analysis. Samples were stored at -70°C prior to analyses.

Receptor analyses

All samples were analysed for oestrogen (ER) and progesterone (PgR) receptors by the dextran charcoal method [4]. A positive receptor status was accorded to those tumours with either an ER or PgR of 10 fmol/mg protein or greater.

DNA analyses

All samples were analysed on an ICP-22 flow cytometer (Ortho Instruments, Westwood, MA, U.S.A.). Samples were stained for DNA content using ethidium bromide/mithramycin in a single-step staining technique which has been adequately described elsewhere [5]. A coefficient of variation of the G_1 peak between 1.2 and 3% was obtained for all samples. Chicken red blood cells (CRBC) were used as an internal marker and analyses of DNA histograms were carried out as previously described [6, 7].

Analysis of patient data

All patient data were obtained from histopathology records. As many of the specimens came from hospitals outside the Sydney area no attempt has been made to review the histology. All data were retrieved and analysed subsequent to DNA analysis and assignment to a ploidy subdivision. The significance of observed differences was evaluated by analysis of variance and Student's *t* test.

RESULTS

One hundred and twenty-one tissue samples were received, all of which were histologically confirmed as primary breast cancer. Seven of these were judged to be unevaluable by DNA analysis; the other 114 samples were allocated to one of five defined DNA ploidy groups (see below) before any further analysis or retrieval of data took place.

Measurement and distribution of DNA ploidy levels

Our previous studies showed that the G_1 peak CRBC ratio for normal diploid cells (peripheral blood lymphocytes) was 2.90 ± 0.17 [6]. Using this criterion all tumour specimens analysed in this study contained a major population of cells with a diploid DNA content (Fig. 1). In 21% of tumours this was the only cell population present (CRBC ratio 2.87 ± 0.12). In the majority of specimens, however, additional peaks representing aneuploid tumour cells were found and in these the diploid peak was assumed to represent only normal diploid cells. Previous studies from this laboratory [8] of other tumour types have suggested that this assumption holds in most instances. The proportion of diploid cells present in such instances varied from about 10 to more than 70%.

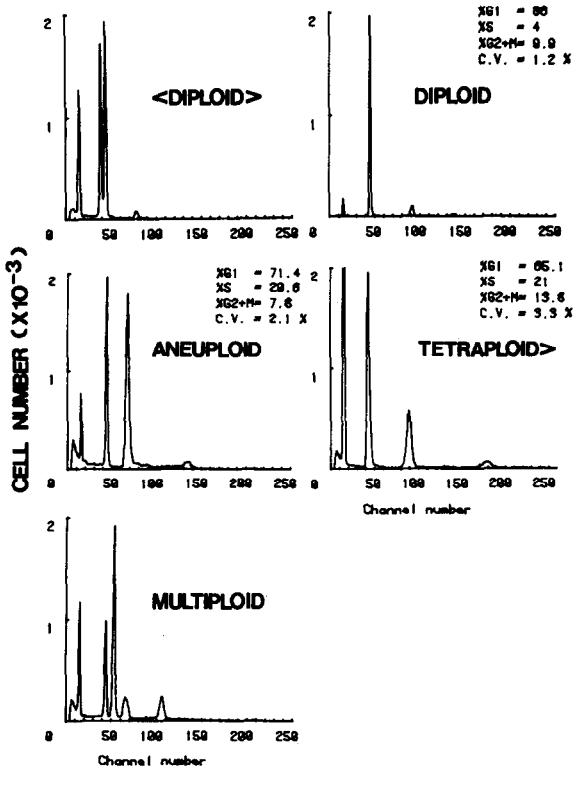

Fig. 1. DNA distributions of primary breast tumours showing representative samples of each DNA ploidy class described in this study. Channel number, relative fluorescence intensity which is directly proportional to DNA content. Number of cells are shown on the ordinate. The peak between channels 10 and 20 in each histogram represents chicken red blood cells which are used as an internal biological standard. The analysis shown for single aneuploid and tetraploid tumours are for the tumour population only. C.V., coefficient of variation of G_1 DNA peak.

Aneuploid tumours were sub-divided as follows: near-diploid (12%) for a DNA content of less than diploid or not more than 10% greater than diploid; single aneuploid (42%, CRBC ratio $= 4.86 \pm 0.51$) for greater than 10% diploid but less than tetraploid; tetraploid (9%, CRBC ratio $= 5.74 \pm 0.16$) for those tumours with a DNA content twice diploid or greater; and multiploid (16%) for tumours where in addition to the diploid peak more than one aneuploid peak could be observed (Fig. 1). It should be noted that the CRBC ratio for diploid cells present in aneuploid tumours (2.88 ± 0.09) was identical to that for purely diploid tumours.

Tumour type

The distribution of tumour types found in this study is shown in Table 1. The majority of tumours examined were infiltrating ductal carcinomas, and while a 90% incidence for this type of tumour is higher than expected, an earlier study reported a similar frequency of 87% [9]. Some histological types such as intraductal carcinomas were almost certainly under-represented because their small size did not yield sufficient spare tissue for this study. It is of interest to note, however, that of the 11 tumours not identified as infiltrating ductal 45% were diploid, compared with 18% diploid tumours for the remainder of the population. Due to the small numbers involved, however, this difference was not significant.

Table 1. Distribution of tumour types

Histology	No.	% diploid
Infiltrating ductal	103	18
Lobular	3	45
Papillary	2	
Medullary	3	
Colloid	1	
Pagets disease and intraductal ca.	1	
Infiltrating mucoid	1	

Tumour size and nodal involvement

Tumour size was obtained by direct measurement of surgical specimens from 111 patients. Twenty-two percent were less than 2 cm (T1), 56% were between 2 and 5 cm (T2) and 22% were greater than 5 cm (T3). Assessment of the involvement of axillary lymph nodes was also obtained from 94 patients by pathologic examination, and the data grouped as nodes negative (48%), <4 nodes positive (23%) and 4+ nodes positive (29%). As can be seen from Figs 2 and 3 respectively, no significant correlations between tumour size or degree of nodal involvement and DNA ploidy level was observed.

Receptor content

All specimens were analysed for both oestrogen and progesterone receptors, with 10 fmol/mg protein or greater being taken as either ER^+ or PR^+. Using this criterion 61 and 54% of tumours were ER^+ and PR^+ respectively. No significant correlation between ploidy and receptor content was found (Fig. 4), although there was a slight tendency for diploid tumours to be receptor-positive. Sixty-nine percent of diploid tumours were receptor-positive, as compared to 58% for all aneuploid tumours. Similar observations were made when the data were re-analysed using absolute values for receptor levels. For aneuploid

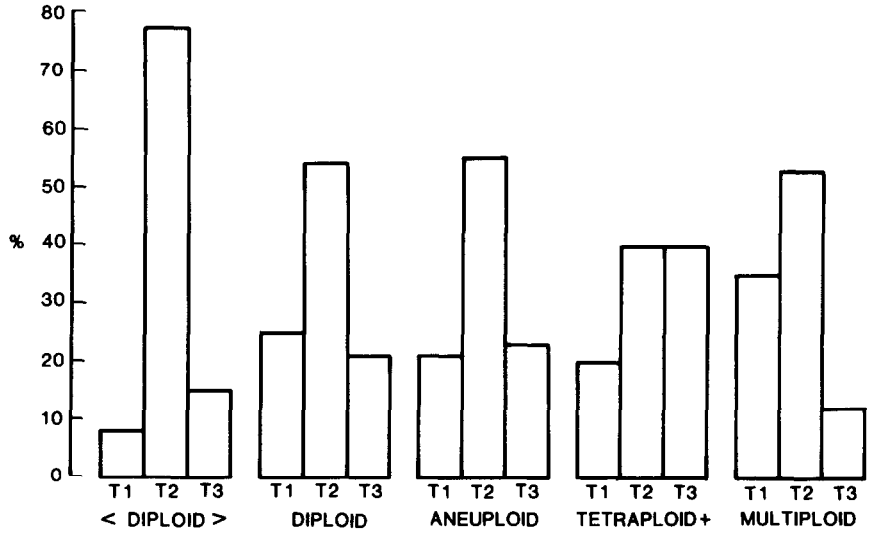

Fig. 2. Distribution of tumour size with DNA ploidy level. Tumour sizes were classified as: T1, <2 cm; T2, between 2 and 5 cm; T3, >5 cm. The ordinate shows the percentage of tumours within each classification.

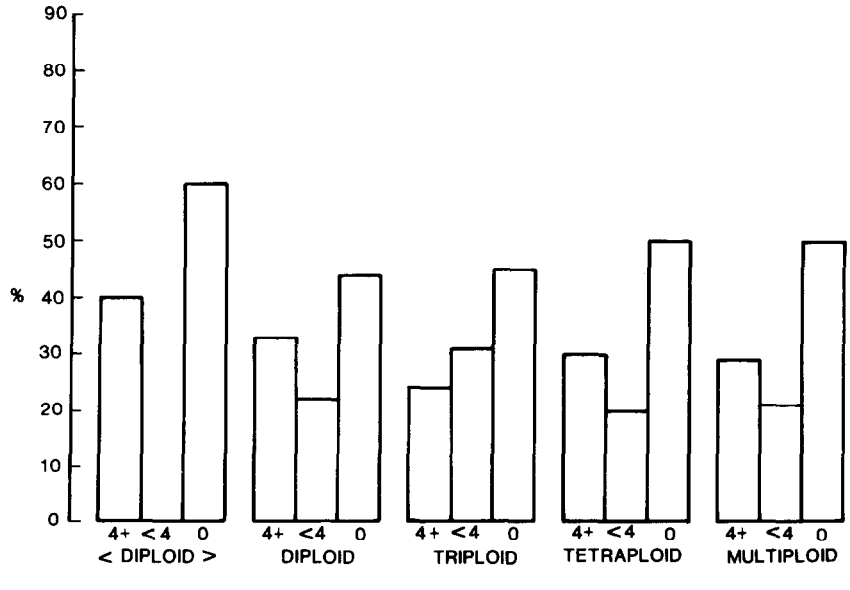

Fig. 3. Distribution of degree of axillary nodal involvement with DNA ploidy. Nodal involvement was assessed by surgical examination.

tumours no significant correlation was observed between receptor status and the proportion of diploid cells present in the population.

Proliferative activity

The proportion of cells in S phase was measured in 63 tumours. Overlapping of sub-populations was the major reason for omitting tumour samples from this analysis. This included occasions where diploid 'normal' cells interfered with the analyses of S phase either by being unresolvable for analytical purposes from the G_1 peak of near-diploid tumours or by contributing significantly to the S phase of aneuploid tumours in specimens where 'normal' cell contamination was particularly high. Thus all diploid and tetraploid tumours were analysed but only 50% of near-diploid and 65% of single aneuploid tumours. No analysis was attempted with the multiploidal tumours. Changes in % S phase associated with DNA ploidy are shown in Fig. 5. The association of a low (≤10%) S phase with 81% of all diploid and near-diploid tumours compared to only 22% of single aneuploid and tetraploid tumours was highly significant ($P = 0.0001$).

Age

Patient age was obtained for all but 2 cases. The distribution and mean age of each ploidy group and for the whole population is shown in Fig. 6.

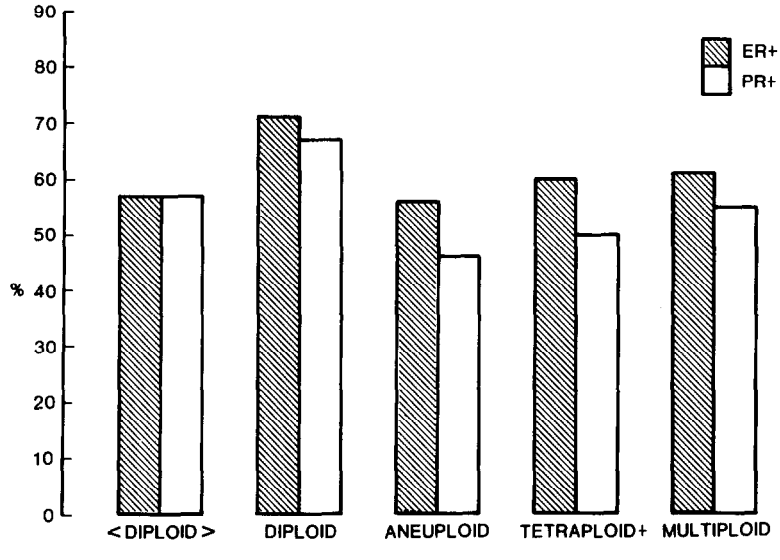

Fig. 4. Distribution of oestrogen (ER) and progesterone (PR) receptor status with DNA ploidy. A lower threshold of 10 fmol/mg *protein was used to determine receptor positivety in all cases.*

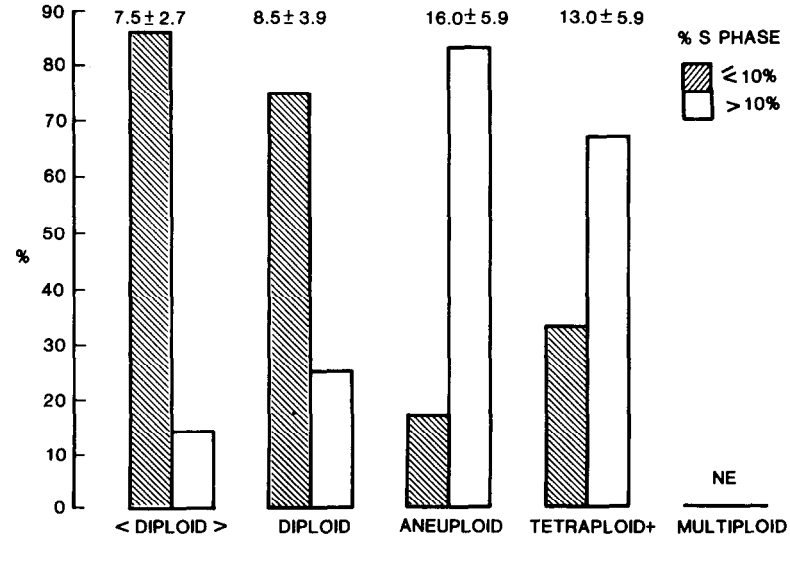

Fig. 5. Changes in proliferative activity with DNA ploidy. Proliferative activity was assessed in 55% of tumours (see text) from computer analysis of the DNA content distributions. Bars show percentage of tumours within each ploidy group with an S phase of greater than or equal to and less than 10%. *The mean percentage of S phase* ±1 *S.D. from the mean is also shown for each group.*

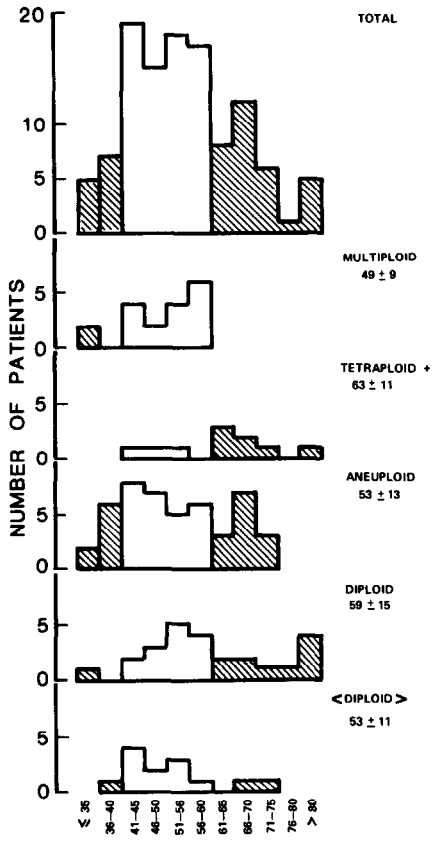

Fig. 6. Distribution of patient age with DNA ploidy and for the population as a whole. Bars show numbers of patients. Shading indicates <40 yr *and* >60 yr. *The mean age* ± 1 *S.D. is also shown for each group.*

Significant differences were noted between mean ages for tetraploid vs all other aneuploid tumours (63 ± 11 vs 53 ± 11; $P = 0.025$) and for multiploid compared to all other tumours (49 ± 9 vs 55 ± 13; $P = 0.024$). In addition, the distribution of age in multiploid tumours was found to differ significantly from the whole population ($F_{4,109} = 2.99$, $P < 0.05$).

DISCUSSION

The distribution of diploid (21%) and aneuploid (79%) tumours in this study is in agreement with a number of previous studies (for a review see [10]). Within the aneuploid tumours, however, we identified a substantial subpopulation of multiploid tumours (16%), where more than one aneuploid peak was present in addition to a diploid peak. Multiploid tumours of this nature have been identified previously [11, 12], albeit at a somewhat lower frequency. Great care was taken during this study to remove any possible staining artifacts which might contribute to this result. The existence of tumours containing a diploid tumour clone in addition to an aneuploid clone must also be considered. In this study where a single anueploid DNA peak is present we have assumed that the diploid peak represents only normal cells. This is in keeping, generally, with observations from our own laboratory [8] and from others [11, 13], where normal cell contamination of tumour specimens of up to 90% has been reported [13]. However, in our previous study, which examined intact cells as opposed to isolated nuclei as used here, one tumour (a seminoma) was identified as containing both diploid and aneuploid tumour cells. Similar observations have been made in mammary carcinomas in studies using single-cell cytophotometry [1]. Thus the incidence of multiclonal mammary carcinomas might well be higher than that reported here.

No significant correlation between ploidy level and tumour type, tumour size, nodal involvement or receptor status was observed in this study. There was, however, a tendency for diploid tumours to be receptor-positive and to belong to a histologic grouping with better prognosis. These findings are generally in agreement with a number of similar recent studies [12, 14, 15], although some of these have found significant correlations between ploidy, tumour type and receptor status [14].

A highly significant correlation between ploidy level and proliferative activity as measured by the proportion of cells in S phase was found, with diploid and near-diploid tumours having a relatively low number of cells in S phase (8.0 ± 3.3%) compared with single aneuploid and tetraploid tumours (15.0 ± 5.9%). Although these findings are in agreement with previously published studies [15], it is possible that the values for S phase in diploid tumours have been artificially lowered by the presence of normal diploid cells, in particular peripheral blood lymphocytes, which would contribute to the G_1 tumour peak but are generally associated with a rather low S phase [16]. In contrast, values of S phase for aneuploid tumours could be increased by the overlapping S and $G_2 + M$ phases of the 'normal' diploid cell component. In an earlier examination of the proliferative status of breast carcinomas using thymidine labelling indices a significant correlation between high labelling indices and early relapse was found [17]. It will be interesting to see if such a relationship is subsequently found in this study, where proliferative activity has been assessed by flow cytometry.

When the data were analysed with regard to patient age a number of significant trends were found. Analyses of the aneuploid group showed a marked tendency for ploidy levels to increase with age of patient, with a mean of 53 ± 11 yr for the near-diploid to less than tetraploid groups

compared with 63 ± 11 yr for the tetraploid or higher group ($P = 0.025$). A similar trend has been previously noted with cancer of the cervix [18]. However, the most striking feature was that 89% of the multiploid tumour group were aged between 40 and 60 yr compared to 56% within that age group for the remainder of the population ($P < 0.05$). Although the precise menopausal status of these patients is not known, the clustering of multiploidal primary tumours in the 40- to 60-yr age ranges suggests that this phenomenon may be related to the well-known refractoriness of recurrent breast cancer to endocrine therapy during the perimenopausal period [19] since the probability of these tumours containing a hormonally insensitive sub-clone might well be greater.

Although at this time no data regarding recurrence or outcome of subsequent treatment are available for this study, the results presented here suggest that measurement of cellular DNA content by flow cytometry could be potentially useful in detecting high-risk sub-populations independent of oestrogen and nodal status.

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