Cellular DNA content—a stable feature in epithelial ovarian cancer

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Detailed flow cytometric analysis of cellular DNA content was performed on neoplastic tissue from 33 patients with malignant common epithelial ovarian tumours in order to investigate the intratumoral stability of ploidy and proliferative fraction. There was a remarkable stability, both spatial and temporal, in the DNA pattern for any particular tumour. Of 24 tumours that were analysed in multiple areas tumour ploidy was found to be a stable marker in all but 3 cases where regional variations were evident. In 9 patients serial analyses were performed on tumour obtained either at initial diagnosis (6 patients) or second look laparotomy (3 patients) and then some time later (7–17 months) at relapse or death and in all cases the tumour ploidy remained unchanged. In addition, 10 ovarian carcinomas established in nude mice have maintained a DNA content during serial passage similar to that of the original implanted tumour. In contrast in 50% of tumours that were evaluable for S-phase analysis we demonstrated a considerable intratumoral variability in the S-phase fraction.

We conclude that cellular DNA content is a stable feature of ovarian carcinoma while S-phase fraction is commonly subject to intratumoral variation.

The flow cytometric determination of cellular DNA content has been shown to be of prognostic value in a number of tumour types (Bunn et al., 1982; Wolley et al., 1982) and we have recently reported that tumour ploidy was an independent prognostic variable and the major determinant of survival in patients with advanced ovarian cancer (Friedlander et al., 1983a). The value of a single estimation of tumour ploidy would be clearly limited however if tumours commonly exhibited a variation in ploidy within different regions of the primary tumour or its metastases. Such variability has been reported in colonic cancer (Petersen et al., 1981) and small cell lung cancer (Vindelov et al., 1980) but there are no studies that have addressed the stability of cellular DNA content as determined with flow cytometry in ovarian tumours. We report the results of a study investigating the frequency of intratumoral heterogeneity of cellular DNA content and proliferative fraction (S-phase) in ovarian carcinomas.

Materials and methods

Tumour specimens for flow cytometric analysis and histological examination were obtained from 33 patients with ovarian carcinoma and multiple areas (mean 4, range 2–12) were biopsied from either the primary tumour alone (8 FIGO I and II tumours) or from the metastatic sites as well when the patient had advanced stage disease (16 patients). Particular attention was paid to include biopsies from the peripheral and central regions of a tumour mass when possible. In an additional 9 patients it was possible to analyse tumour ploidy sequentially on specimens obtained at either initial diagnosis (6 cases) or from residual tumour found at second look laparotomy (3 cases) and then at subsequent relapse. Operative specimens were received fresh from the operating theatres and tumour tissue was subdivided in the pathology department for flow cytometry and histological examination. DNA flow cytometry was usually performed within 2h of operative removal of tumour from the patient but if not, samples were stored at −70°C prior to analysis. Ascitic or pleural fluid specimens were examined cytologically and flow cytometrically immediately following aspiration.

Flow cytometry

DNA content All specimens were analysed on an ICP 22 flow cytometer (Ortho-Instruments, Westwood, MA). Specimens were processed and stained for DNA content with ethidium bromide and mithramycin in a single step staining technique that has been fully described elsewhere (Taylor, 1980). Briefly, for solid tumours 250 μl of a 1% Triton-x-100 solution containing 400 μg ml⁻¹ ethidium bromide and 250 μl of a 1% triton solution containing 125 μg ml⁻¹ mithramycin and 75 mM magnesium chloride were added to 2ml of RPMI
1640 medium containing 10% foetal calf serum. A
2 ml aliquot of this staining solution plus 200 μl
chicken red blood cells (CRBC; 10^6 ml^-1) were
added to a small piece of tumour tissue
(5 mm × 5 mm) in a petri dish and the tissue was
finely disaggregated with scalpel blades. A further
2 ml of staining solution were added, the solution
gently pipetted several times and the preparation
filtered through an 80 μm nylon mesh filter. This
preparation results in a suspension of isolated
nuclei from which almost all cytoplasm has been
removed. RNA-ase (ribonuclease Type IA, Sigma
Chemical Company) was added to the suspension
just prior to analysis to give a final concentration
of 1 mg ml^-1.

Chicken red blood cells (CRBC) were used as an
internal marker, as the ratio of the G1 DNA
content of human diploid cells to the DNA content
of CRBC is highly reproducible (2.9 ± 0.17) under
the staining conditions used (Taylor & Milthorpe,
1980). Using this standard all the tumour specimens
analysed contained a population of cells with a
diploid DNA content. When this was the only cell
population present the tumour was classified as
diploid, while tumours which had evidence of an
additional G1 peak were classified as aneuploid.
Where in addition to the diploid G1 peak there was
clear evidence of more than one aneuploid G1 peak
the tumour was classified as multiploid. Ploidy was
further quantitated by the DNA Index (DI) which
represents the relative DNA content of the
aneuploid G1 cells in comparison to diploid cells
(Barloge et al., 1980). Thus, a DI of 1 is
synonymous with a normal diploid DNA content.
The mean coefficient of variation of the diploid or
aneuploid G1 peak was 2.7% in all tumours studied
(range 1.2–5.1).

In order to determine the sensitivity of the
method of detecting an aneuploid tumour
population, normal human lymphocytes (diploid)
were admixed with an aneuploid ovarian cell line
(DI = 1.59). Varying amounts of aneuploid cells
were mixed with human lymphocytes to give a final
cell concentration of 10^6 ml^-1 with an aneuploid
cell content ranging from 0.5%–95%. The cells
were stained with ethidium bromide and mithramycin as described above.

Xenografted tumours
Balb/C nu/nu strain female nude mice were
inoculated with fragments of ovarian tumour tissue
as previously described (Van Haafken–Day et al.,
1983). All tumours had DNA analysis and
histological examination performed prior to
implantation and with each serial passage.

Results
Comparison of tumour ploidy within primary tumour
and metastases
Twenty-four patients had tumour ploidy
determined either from different sites within the
primary tumour alone (8 cases) or from the
metastatic sites as well in those patients with
advanced stage disease. Thirty-three percent of the
patients had diploid tumours and 67% had
aneuploid tumours. Figure 1 outlines the
distribution of DNA content and demonstrates a
clustering of tumour cells about a diploid and
triploid-tetraploid mode. The majority of tumours
(87%), exhibited a stable DI in all sites analysed
(Figure 2) and this regional stability of DNA
content was also evident in 2 multiploidal tumours
(Figure 3). The 3 exceptions (all Stage III tumours)
included a tumour which was initially classified as
diploid but had an additional tetraploid G1 peak
demonstrated in one region and two aneuploid
tumours which showed regional variations of
aneuploidy (Figure 4). Only one of these tumours
had concomitant regional histological differences
which correlated with the ploidy variations (a
mixed epithelial carcinoma with adenosquamous
and typical serous carcinoma in different areas).

Although the DI was stable in different areas of
most tumours there were nevertheless often regional
variations (for examples, see Figure 4) in the actual
proportion of aneuploid to diploid cells. We
determined the sensitivity of flow cytometry in
detecting aneuploid populations and under
controlled conditions demonstrated that a 1%
concentration of aneuploid cells admixed with
normal human lymphocytes could be identified
(Figure 5).

Sequential analysis of DNA content
Nine patients had DNA analysis performed on
sequential tumour specimens. The initial specimen
was obtained either at initial diagnosis or at a
second look laparotomy where residual tumour
remained after chemotherapy. Tumour DNA
analysis was repeated in all patients at a subsequent
relapse or death with a mean interval of time
Figure 1 Frequency histogram showing the distribution of ploidy in tumours from 24 patients with ovarian cancer. Ploidy is expressed as DNA index (DI) which represents the relative DNA content of tumour G1 to diploid G1 cells. All tumours analysed contained a population of cells with a diploid DNA content and when this was the only cell population present the tumour was classified as diploid (DNA index 1). When there was an additional G1 peak the tumour was classified as aneuploid and in such cases the diploid component was assumed to be made up of normal cells. Five tumours had 2 aneuploid G1 peaks while the rest had a single tumour G1 peak.

between the first and second DNA analysis of 9 months (range, 7–15 months). Two patients initially had diploid tumours and 7 had aneuploid tumours and the DI of all tumours remained unchanged at the time of relapse or death (Table I).

**Xenografted ovarian tumours**

Ten epithelial ovarian carcinomas were established directly in nude mice and histological examination and DNA analyses were performed on tumour tissue just prior to implantation and repeated at each subsequent passage. The DI (Table II) and histological features have remained essentially unchanged during the serial passage of all tumours to date (median time = 12 months).

**Table I** Comparison of DNA index at diagnosis and at time of relapse or death

| Patient | Initial DNA index | DNA index at relapse |
|---------|-------------------|---------------------|
| 1       | 0.85              | 0.83                |
| 2       | 0.9               | 0.85                |
| 3       | 1                 | 1                   |
| 4       | 1                 | 1                   |
| 5       | 1.17              | 1.15                |
| 6       | 1.31              | 1.31                |
| 7       | 1.73              | 1.73                |
| 8       | 1.78              | 1.77                |
| 9       | 1.83              | 1.79                |

**Table II** Comparison of DNA index of ovarian tumour xenografts passed in nude mice with the DNA index of original tumour

| Tumour | Original DI of fresh tumour | DI after passage in nude mice | No. of passages |
|--------|----------------------------|-------------------------------|----------------|
| 1      | 1.64                       | 1.73                          | 4              |
| 2      | 2.60                       | 2.53                          | 2              |
| 3      | 0.86                       | 0.92                          | 6              |
| 4      | 1.45                       | 1.60                          | 4              |
| 5      | 1.35                       | 1.40                          | 3              |
| 6      | 1.33                       | 1.42                          | 3              |
| 7      | 2                          | 2                              | 5              |
| 8      | 1.47                       | 1.52                          | 6              |
| 9      | 1                          | 1                              | 3              |
| 10     | 1.72                       | 1.9                            | 3              |
populations (50%) and had the content for Of the intense interest. We have evidence of an additional tetraploid component in one area and 2 aneuploid tumours which exhibited regional variations of aneuploidy tumour ploidy was a stable marker. S-phase analysis was possible in all diploid tumours but only in 50% of aneuploid tumours as overlapping of cell populations precluded accurate analysis. A 40% or greater variation of S-phase was evident within different areas of the same tumour in half the cases analysed.

Regional S-phase variation

Of the 24 tumours studied for regional variations in DNA content all 8 diploid tumours were evaluable for S-phase analysis while overlapping of cell populations precluded an S-phase estimation in half the tumours with aneuploid cells. Diploid tumours had a mean S-phase of 6.6% (range, 3.4–16.2%) and aneuploid tumour cells a mean S-phase of 17.3% (range, 3.7–33.6%). In 8 of the tumours (50%) there was a 40% or greater variation of S-phase (Figure 2) in different sites.

Discussion

Flow cytometric analysis allows tumour DNA content and proliferative activity to be determined rapidly and precisely and their value as possible objective parameters reflecting tumour biology is of intense interest. We have shown that cellular DNA content is an independent prognostic variable and a major determinant of survival in advanced ovarian cancer (Friedlander et al., 1983a) and similar findings have been reported in other malignancies (Bunn et al., 1982; Wolley et al., 1982). There is however, some evidence that regional variations in tumour ploidy are common in colonic carcinomas (Petersen et al., 1981) and small cell lung cancer (Vindelov et al., 1980) and the heterogeneity of such tumours casts doubts on the value of a single estimation of ploidy as a prognostic index for all tumour types. We have therefore studied the stability of cellular DNA content in common epithelial ovarian cancer.

While ovarian tumours as a group are widely heterogeneous with respect to DNA content (Atkin, 1970; Friedlander et al., 1983b) with early stage ovarian cancers being commonly diploid and

![Figure 2](image-url) Histogram to demonstrate the relative regional stability of tumour ploidy and relative regional variation of S-phase fraction in 24 ovarian cancers sampled in different areas of the tumour. Apart from 1 predominantly diploid tumour which had evidence of an additional tetraploid component in one area and 2 aneuploid tumours which exhibited regional variations of aneuploidy tumour ploidy was a stable marker. S-phase analysis was possible in all diploid tumours but only in 50% of aneuploid tumours as overlapping of cell populations precluded accurate analysis. A 40% or greater variation of S-phase was evident within different areas of the same tumour in half the cases analysed.

![Figure 3](image-url) Demonstration of stability of DNA content in a multiploid tumour. There is evidence of 2 aneuploid clones in both the primary tumour and omental metastases. Note the resolving power of the instrument in discriminating the 2 diploid peaks. Channel no., relative fluorescence intensity (DNA content). Ordinate, number of cells.
advanced stage carcinomas commonly aneuploid, we demonstrate in this study that within an individual tumour intratumoral variations of ploidy are uncommon, occurring in only 13% of cases. These findings support those of an earlier study where, using Feulgen microspectrophotometry, ovarian cancers were shown to have a stable DNA content within different sites (Atkin, 1970). Further evidence of the stability of cellular DNA content in epithelial ovarian cancer is provided by finding that in ovarian tumours established in nude mice the DI remains unchanged during serial passage. The mean variation of the DI before and after passage in nude mice was 4.5% (range, 0–10%). This is in keeping with the variation that can be expected by possible staining and instrumental variation (Taylor & Milthorpe, 1980).

It was possible to determine the stability of cellular DNA content during tumour progression in 9 patients by analysing tumour specimens obtained at initial diagnosis or at a second look laparotomy and then again 7–17 months later at the time of relapse or death and in all cases the DI remained unchanged. All these patients had initially responded to chemotherapy with cis-platinum and/or chlorambucil and had later relapsed. It is interesting that there was a reappearance of clones with the same DNA content as that found prior to treatment, which suggests some stability of the genome. There could however be chromosomal variability associated with disease progression but masked either by minor changes undetectable with flow cytometry (Barlogie et al., 1977) or because chromosomal number variability may be due to a DNA packaging defect which would not be reflected by a change in the DNA content (Kraemer et al., 1971). Only a small number of cases have been reported where serial karyotyping and banding studies have been performed following the progression of ovarian tumours and such studies show that the karyotype remains basically unchanged during tumour progression with changes, when present, representing a variation in the theme already observed in the original tumours (Atkin, 1970; MacKillop et al., 1983; Sandberg, 1982).

While the DI remains constant within a primary tumour and its metastases the proportion of aneuploid cells often varies from one region to another. This feature has been interpreted by some (Nervi et al., 1982) as evidence of intratumoral heterogeneity but it is likely that these variations reflect different degrees of admixing of normal diploid cells with aneuploid tumour cells rather than changes of biological significance. We cannot rule out entirely the possibility that cells with a diploid DNA content in tumours with bimodal

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**Figure 4** DNA histograms of a tumour that exhibited a regional variation in aneuploidy. Channel number represents fluorescence intensity which is directly proportional to DNA content. The number of cells counted are shown on the ordinate. The first peak corresponds to chicken red blood cells (CRBC) which acts as an internal biological standard and is set in channel 10. The next major peak represents the diploid G1 peak. Note that in subset c only one aneuploid population is evident while in subsets a and b two aneuploid populations are present but the relative proportions vary.
DNA distributions could represent an additional tumour clone but a number of studies have shown that in these circumstances the diploid cells usually represent normal cells (Barlogie et al., 1978; Perez et al., 1981).

We determined the sensitivity of flow cytometry to detect aneuploid tumour cells admixed with normal cells under controlled conditions and showed that as low as a 1% content of tumour cells could be detected. However, for practical purposes, we suggest the limit of detection of an aneuploid cell population is between 5% and 10% of the cells analysed due to the presence of background debris and the possible masking effect of the S-phase and G₂+M components of diploid cells. Histological examination of all specimens analysed is therefore necessary to ensure that they contain adequate and representative portions of tumour tissue. This assumes particular importance in diploid tumours where the finding may have important prognostic implications, as these tumours have a more favourable natural history than aneuploid tumours (Atkin, 1970; Bunn et al., 1982, Friedlander et al., 1983b; Wolley et al., 1982). The actual degree of aneuploidy (i.e. DI of tumour) or the presence of multiploidy has not been demonstrated to date to be of significance and although it has been suggested by some that multiploid tumours may have a more aggressive natural history (Taylor et al., 1983; Vindelov et al., 1980), we could not confirm this in patients with advanced ovarian cancer. All 3 patients who had regional variations in ploidy in this study relapsed within 6 months of diagnosis and it is possible that an unstable DNA content identifies high risk patients.

The S-phase as determined flow cytometrically has been reported to be a reflection of the aggressiveness of tumour behaviour and to be of prognostic significance (Costa et al., 1981). The S-phase can be determined in all diploid tumours but
only in \(~50\)% of aneuploid tumours because of overlapping of tumour populations. While we and others have demonstrated that diploid tumours have a lower S-phase than aneuploid tumours, it is possible that the values for S-phase in diploid cells have been artificially lowered by the presence of normal non-cycling diploid cells e.g. lymphocytes (Dosik et al., 1980), while aneuploid tumours could have a higher S-phase because of the presence of diploid doublets and tetraploid normal cells. In this study we found that up to 50% of tumours showed a significant regional variation in the S-phase fraction (\(>40\)% variation in different areas). The reasons for this variability are not known but could relate either to different proliferative states among tumour cells in different nutritional environments, or to variable contamination of tumour cells by normal non-cycling cell populations. The value of a single S-phase estimation is therefore clearly limited in the light of these findings, as are data relating to the prognostic value of S-phase in ovarian cancer.

We conclude that DNA content is a stable feature in most cases of epithelial ovarian cancer exhibiting constancy within the primary tumour, its metastases and during subsequent tumour progression. On the other hand, the S-phase fraction is often subject to considerable intratumoral variation. These results cannot necessarily be extrapolated to other tumour types where similar studies are required to establish the representative value of a single estimation of tumour DNA content or proliferative fraction.

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