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Argyrophilic Nuclear Organiser Region Counts in Locally Advanced Breast Carcinoma Treated by Chemotherapy before Surgery

Wladimir V. Bogomoletz, Christiane Pourny and Brigitte Didier

The value of argyrophilic nuclear organiser region (AgNOR) counts in assessing histologically the effects of combination chemotherapy given to eleven patients with locally advanced breast cancer before mastectomy was studied. AgNOR counts were significantly reduced ($P < 0.001$) in the post-chemotherapy, surgically excised residual tumour specimens compared with the initial diagnostic biopsy specimens. AgNOR counts could be used to monitor the effects of chemotherapy on breast cancer.
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NUCLEOLAR organiser regions (NORs) have attracted much attention in tumour pathology [1–4]. NORs are chromosomal segments containing encoded ribosomal RNA genes, and are likely to play a major role in nucleolar activity, thus contributing to cell proliferation [5]. The number of NORs detected in neoplastic cells may hence reflect tumour cell kinetics, with possible prognostic implications. Being intimately associated with argyrophilic proteins, NORs can be visualised as AgNORs in routine paraffin sections by a modified silver technique [6]. There are significant correlations between AgNOR counts, Ki67 immunostaining and DNA flow cytometry in human breast cancer specimens [7–9]. Our aim was to investigate the usefulness of AgNOR counts in assessing histologically the effects of combination chemotherapy in patients with locally advanced breast cancer before mastectomy.

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PATIENTS AND METHODS

Eleven cases of infiltrating carcinoma of the breast were selected from the files of the Institut Jean Godinot on the following basis: (1) locally advanced breast cancer staged T_3–T_4 (M_0 and irrespective of N status), N_2–N_3 (M_0 and irrespective of T status) or with inflammatory carcinoma (M_0 and irrespective of T and N status); (2) each patient had had one initial diagnostic biopsy of her breast tumour (Tru-cut, drill or incisional biopsy); (3) following histological confirmation of the invasive neoplastic nature of the mammary lesion, each patient had received four cycles of combination chemotherapy (doxorudicin 30 mg/m^2 a total of 50 mg or less on day 1, vincristine 1 mg/m^2 to a total

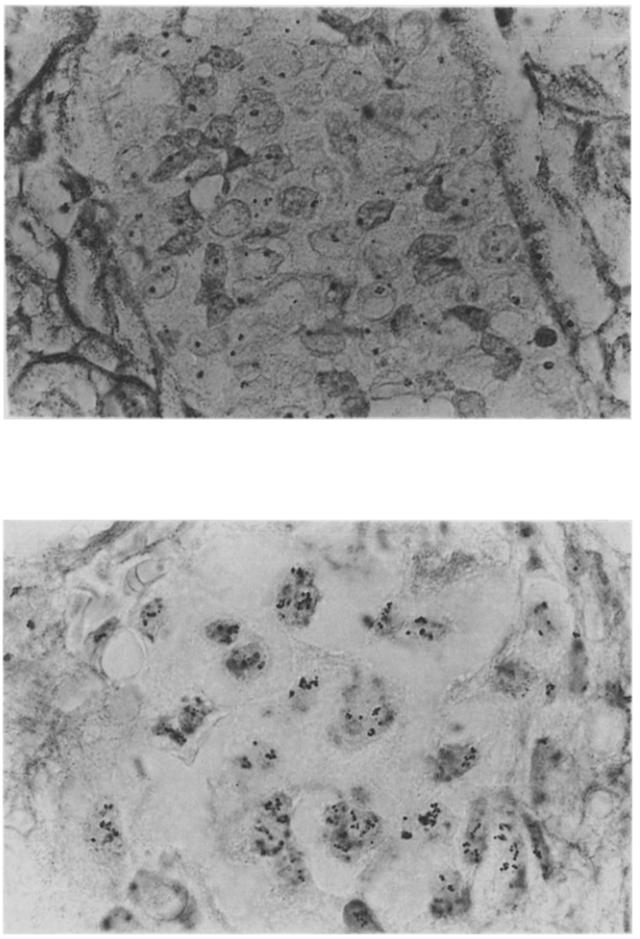

Fig. 1. AgNOR staining. Upper=initial diagnostic biopsy specimen of infiltrating duct carcinoma before combination chemotherapy (case 2); many nuclei contain large numbers of AgNORs. Lower=post-chemotherapy surgically excised residual tumour specimen with decreased numbers of AgNORs in nuclei (×434).

of 1.5 mg or less on day 2, 5-fluorouracil 400 mg/m^2 and cyclophosphamide 300 mg/m^2 from days 3–6 [after the first cycle, each subsequent cycle was repeated on day 29]; (4) 3–4 weeks following the full course of combination chemotherapy, a Patey radical mastectomy had been done (all the surgical breast specimens had contained residual tumour macroscopically and histologically); and (5) all the tissues from the initial biopsies and the subsequent radical mastectomies had been fixed in 10% formol saline and processed routinely in paraffin. All the original haematoxylin–eosin stained slides were carefully reviewed for histological changes. Patients' notes were also retrieved and checked.

AgNOR staining

The paraffin blocks of the eleven initial biopsy specimens and those of one representative area of residual cancer found in the subsequent mastectomies were recut at 4 μm. After overnight drying at 60°C, the sections were dewaxed in xylene for 1 h at 37°C. The slides were then immersed in succession in 100° ethanol for 1 h and in Clarke's fixative for 10 min. The sections were rehydrated through descending alcohols. The colloidal silver staining solution was prepared for immediate use by mixing two parts of a 50% silver nitrate aqueous solution with one part of 2% gelatin in 1% aqueous formic acid. The staining time was 30–35 min in the dark.

AgNOR counting

To minimise bias, the original reference number on the slides was erased and replaced by a different code. Appropriate tumour areas free from inflammation, necrosis and haemorrhage were selected and marked by a pathologist (W.V.B.) for AgNOR counting; the AgNOR counts were done by a trained technician (B.D.). In all the slides, the nuclei of 100 neoplastic cells contiguous to one another were assessed with an oil immersion objective. AgNORs were seen as intranuclear black dots of varying size (Fig. 1). All individual black dots within the nuclei were counted. Where two or more black dots were so close that the precise number of individual dots could not be counted, this was recorded as one dot. Two separate counts were made on each slide and the mean between two counts was calculated. AgNOR counting of 100 neoplastic cells took 20–45 min.

RESULTS

The eleven patients were aged 28–74 (mean 53.8). Clinically, after receiving the full dose of combination chemotherapy and before undergoing surgery, all eleven had shown a 50% or more reduction in breast tumour volume. The diameter of the residual tumour contained in the eleven mastectomy specimens after chemotherapy ranged from 1 to 14 cm (mean 3.86). The corresponding axillary lymph-node status ranged from 0 to 16 metastatic lymph nodes (mean 4.2), the total number of dissected lymph nodes ranging from 3 to 22 (mean 12.5). Histological study showed infiltrating duct carcinoma in nine cases and infiltrating lobular carcinoma in two. There were no significant histological features which could differentiate between untreated and treated tumour tissue.

The mean number of AgNOR dots per malignant neoplastic cell is shown in Table 1. The differences in the AgNOR counts

Table 1. AgNOR counts in locally invasive breast carcinoma before and after combined chemotherapy

Case No	Mean number of AgNOR dots per malignant neoplastic cell: Initial biopsy specimen	Residual tumour specimen
1	6.87	3.09
2	17.94	6.12
3	13.94	8.85
4	11.66	7.64
5	14.44	8.22
6	11.58	10.74
7	11.54	4.92
8	8.19	5.63
9	16.67	7.35
10	11.55	5.46
11	10.24	7.88
Total (mean, S.D.)	12.23 (3.20)	6.90 (2.11)

before and after chemotherapy were statistically significant (paired t test, $P < 0.001$).

After the initial preoperative combination chemotherapy followed by mastectomy, all but one of the eleven patients received varying regimens of adjuvant chemotherapy, radiotherapy or hormone therapy. Five patients were alive and free of disease from 6 to 8 years after surgery (mean 6.8). Two patients were alive with bone metastases, both at 5 years after surgery. The remaining four patients died within 1–5 years after surgery (mean 2.75); two of them had widespread disease. No significant correlation was shown between patient's outcome and AgNOR counts before and after chemotherapy.

DISCUSSION

Two methods of assessing tumour cell kinetics are favoured by pathologists: DNA flow cytometry (ploidy and growth phase) and Ki67 immunostaining (Ki67 score). The first method requires the preparation of nuclear suspensions and the use of computer systems and image analysis, while the second method requires frozen sections. AgNOR staining is attracting attention as an alternative tool [1–4]. The procedure does not require expensive equipment or reagents and can easily be done on routine paraffin sections [6]. However, AgNOR counting has some limitations. The actual counting of silver-positive intranuclear black dots representing AgNORs is tedious and time-consuming. The type of tissue fixative [10] and the heterogeneous distribution of cell populations within tumours [11] may influence AgNOR counts.

AgNORs counts in paraffin sections from benign lesions and carcinoma (*in situ* and infiltrating) of the breast has been compared with Ki67 immunostaining [8, 9]. These studies showed a significant correlation between AgNOR counts and Ki67 scores, particularly for infiltrating carcinoma. Therefore AgNOR counts alone may adequately reflect tumour cell proliferation in paraffin sections of invasive breast cancer.

In our study, the mean number of AgNORs per neoplastic cell (mean 12.23) in the initial biopsy specimens of eleven untreated infiltrating (ductal and lobular) carcinomas of the breast was nearer to the mean of 13.77 obtained by Dervan *et al.* [9] than to the mean of 5.46 obtained by Raymond and Leong [8]. Giri *et al.* [7] reported a mean number of 4.22, whereas Smith and Crocker [12] found means of 16.9 and 9.7 for, respectively, ductal and lobular infiltrating carcinomas.

Our study also showed a statistically significant drop in the mean number of AgNORs per neoplastic cell (mean 6.90) in the residual breast tumours in the mastectomy surgical specimens. This drop obviously reflected effects on tumour cell proliferation of the combination chemotherapy given before surgery.

AgNOR counts of tumour cells in paraffin sections might be used to monitor the effects of chemotherapy on breast cancer. One could envisage taking sequential biopsies (tru-cut or drill) of the breast tumour mass before and during combination chemotherapy. AgNOR counts on these specimens would reflect the beneficial effect or not of the drugs on tumour cell kinetics. This method may even provide the oncologist with the possibility of regulating treatment for each patient by determining the best time for surgery or by modifying chemotherapy according to the results of AgNOR counts.

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