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The Role of Nucleolar Organiser Regions as Prognostic Factors in Breast Cancer

Matti J. Eskelinen, Pertti K. Lipponen, Yrjö Collan and Kari J. Syrjänen

Nucleolar organiser regions (NORs) were stained in paraffin-embedded biopsy specimens of 80 female breast carcinomas by the silver (Ag) technique. The patients were prospectively followed up for a mean of 12.4 years (range 11.5–13.3). The number of different types of Ag-NORs was correlated with the histological grade, clinical stage, DNA ploidy, S-phase fraction (SPF) and clinical outcome. Grade III tumours showed higher Ag-NOR counts than low grade tumours. The total number of Ag-NORs ($P = 0.0059$) and the number of dispersed Ag-NOR ($P = 0.0199$) were significantly related to DNA ploidy aneuploid tumours showing higher Ag-NOR counts. The number of aggregated Ag-NORs was predictive ($P = 0.0413$) for the development of metastatic disease during follow-up. On the other hand, crude, cancer-related or recurrence-free survival could not be predicted significantly by the Ag-NORs. The results suggest that the number of Ag-NORs is clearly related to the proliferative activity in breast cancer, but the prognostic value of Ag-NOR counting is inferior to the previously recognised prognostic factors.
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INTRODUCTION

A NUMBER OF well-established prognostic factors have been elucidated by now to predict the clinical course of breast cancer. Such factors include the clinical stage [1–6], histological type [4], histological grade [7–9], hormone receptors [10], parameters measured by quantitative histology [9, 11], mitotic index [9], DNA aneuploidy [12–16] and S-phase fraction (SPF) [14, 16]. Tumour-associated antigens seem to be of some assistance in evaluating the malignancy of breast cancer as well [17–19].

Nucleolar organiser regions (Ag-NORs) represent the loops of DNA actively transcribing to ribosomal RNA (rRNA) [20]. The technique to demonstrate NORs by a simple silver (Ag) staining was described 15 years ago by Goodpasture *et al.* [21]. In 1986, Ploton *et al.* introduced a modification, which made method applicable for formalin-fixed, paraffin-embedded tissue sections [22]. This has prompted a large number of studies on Ag-NORs as possible prognostic factors in a variety of malignant

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tumours, including the female breast cancer [23–25]. However, the current understanding of the possible clinical value of Ag-NORs in breast cancer is still limited [23–25].

The present study was carried out to assess the relationships between Ag-NORs and the other known prognostic indicators of breast cancer. We report the analysis where Ag-NORs were related to the postsurgical stage, histological grade, histological type, DNA ploidy, SPF and survival in a series of prospectively followed up breast cancer patients.

PATIENTS AND METHODS

Patients

We studied 80 consecutive women operated on for a primary breast cancer during a 2-year period between 1975–1976. The mean (S.D.) age of the patients at the time of diagnosis was 58.1 (13.1) years and they were prospectively followed up for a mean of 12.4 years (range 11.5–13.3). Altogether, 41 patients were subjected to a modified radical mastectomy (Patey) [26] in which the major pectoral muscle was preserved, but the minor muscle was removed. 5 patients were subjected to a modified radical mastectomy in which the pectoral muscles were saved, but the muscle fascias were stripped. Simple mastectomy was performed on 34 patients. Axillary lymphadenectomy was included in all treatment methods. None of the patients received chemotherapy preoperatively. Postoperative radiotherapy was given if the tumour was of stage II or higher (n = 37) [1].

Clinical data

For all patients, clinical and pathological data were recorded at mastectomy including the age of the patient, menopausal status, lymph-node status, tumour size and presence of distant metastases. Accordingly, the patients were classified as premenopausal if they were still menstruating at the time of surgery or younger than 52 years. Lymph-node involvement was judged histologically at the time of mastectomy. All lymph-nodes were sliced into 4–5 mm thick sections, embedded in paraffin, and stained with haemotoxylin-eosin. Tumour size was recorded as the maximum diameter in the fresh mastectomy specimen. Distant metastatic localisation was detected by bone and liver radioisotope scan, liver ultrasound, routine laboratory tests reflecting the bone and liver metabolism, and native bone and chest radiography. Metastases were verified by surgical biopsy, when possible. Patients with metastases were given hormonal therapy if the metastatic tumours were hormone receptor positive. The histological typing of the tumours was done according to Azzopardi [27]. The grading was completed according to the grading system of WHO [28] using three grades.

Follow-up

All patients were investigated at the Outpatient Department of Surgery by the same surgeon. During the first year, all patients were examined every 3 months, followed by examinations at 6-month intervals for 72 months, and annually thereafter. Survival and the time elapsed before recurrence were recorded. The mean follow-up time was 12.4 years (range 11.5–13.3 years), and the follow-up was terminated by December 1987.

Methods

The peri-operative biopsy specimens taken before any treatment were fixed in 10% buffered formalin (pH = 7.0), embedded in paraffin, sectioned at 5 μm and stained. The Ag-NOR staining of archival tissue sections (5 μm) was done using the method described by Smith and Crocker [23] with minor modifications

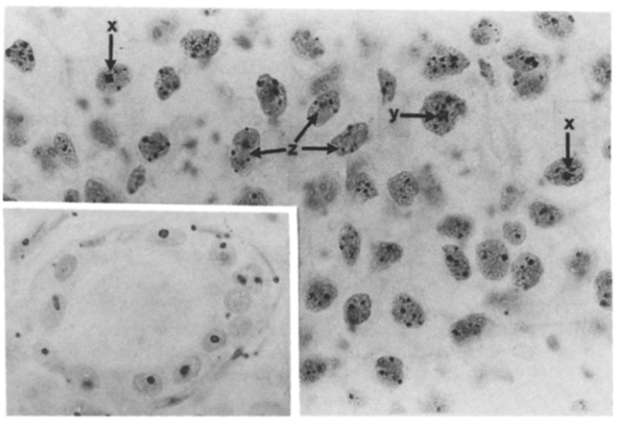

Fig. 1. Typical microscopic images showing large round particles (x), aggregates of Ag-NORs (y) and dispersed Ag-NORs (z). Note the spindle shaped connective tissue cells and normal ductal epithelial cells containing one Ag-NOR/ nucleus (lower left corner). (Magnification ×490).

[29]. In brief, the 5 μm thick paraffin sections were dewaxed in xylene (5 min) and rehydrated through ethanols to distilled deionised water. The Ag-NOR solution was made by dissolving gelatin in 1 g/dl aqueous formic acid at a concentration of 2 g/dl. This solution was mixed (1:2) with 50 g/dl aqueous silver nitrate solution to get the final solution for the staining procedure. This solution was immediately poured over the sections and left for 30 min at room temperature in a dark place. The silver colloid was then washed out using distilled deionised water and the sections were further dehydrated through graded ethanols to xylene and mounted.

For counting the Ag-NORs, the sections were examined under-immersion lens at a total magnification of 1000×. The microscopic fields for analysis were selected at random avoiding the necrotic areas since the staining in these regions is impossible to interpret reliably. In every section, 60 nuclei were examined at random in the centres of six consecutive fields, ten neighbouring nuclei in each. The maximum number of Ag-NORs visible within a nucleus was assessed by focusing the microscope. Vascular endothelial cells and connective tissue cells served as internal controls, usually presenting with one or two small round Ag-NORs/nucleus. Because of the large variation in the size and shape of the Ag-NORs, the following simplifying rule was used in counting: large round particles (LRP: nucleoli?), aggregates of Ag-NORs (A-NOR: deformed nucleoli?), dispersed Ag-NORs (D-NOR) and the total number of Ag-NORs (T-NOR) (Fig. 1).

The flow cytometric DNA analysis of the present study was

Table 1. LRP, A-NOR, D-NOR and T-NOR in different histological grades

Grade	LRP	A-NOR	D-NOR	T-NOR
I (n = 19)	1.14(0.30)	0.18(0.41)	0.88(0.52)	2.20(0.69)
II (n = 46)	1.20(0.34)	0.21(0.31)	0.91(0.54)	2.32(0.72)
III (n = 15)	1.29(0.31)	0.13(0.17)	1.03(0.48)	2.45(0.66)
P*	0.44	0.69	0.69	0.61

Mean (S.D.).
*Analysis of variance.

Table 2. LRP, A-NOR, D-NOR and T-NOR in diploid and aneuploid tumours

DNA ploidy	LRP	A-NOR	D-NOR	T-NOR
Diploid (n = 30)	1.18(0.26)	0.11(0.17)	0.75(0.39)	2.04(0.48)
Aneuploid (n = 50)	1.22(0.36)	0.23(0.37)	1.03(0.57)	2.48(0.76)
P	0.69	0.077	0.02	0.006

Table 3. Crude, cancer-related and disease-free survival related to A-NOR counts

	Survival		
A-NOR values	Crude	Cancer-related	Disease-free
0.00–0.02 (n = 28)	86.8(54.0)	85.9(55.2)	71.2(59.3)
0.02–0.22 (n = 30)	82.2(56.2)	85.4(60.3)	65.1(58.5)
0.22–1.80 (n = 22)	73.0(58.0)	73.0(58.0)	51.4(53.8)
P	0.68	0.70	0.48

done by a standard method as described in detail previously [16]. In statistical calculations, the SPSS/PC$^+$ programme package was used in an Amstrad PC 164OHD20 computer.

RESULTS

The range [mean(SD)] of different types of Ag-NORs per breast cancer cell nucleus were as follows: LRP [0.1–2.2, 1.2(0.3)], A-NORs [0–1.8, 0.2(0.3)], D-NORs [0.1–2.5, 0.9(0.5)] and T-NORs [1.3–3.6, 2.3(0.7)]. The number of Ag-NORs was not significantly related to histological grade of the tumours, as shown in Table 1, although grade III tumours showed higher numbers of Ag-NORs. The Ag-NORs were not related to the histological type of the tumour (76 ductal carcinomas, 4 lobular carcinomas), size of the tumour or axillary lymph-node involvement. The number of A-NORs was significantly related to metastasis (N-stage) (P = 0.0413), the values being lower in patients without metastasis [0.09(0.16)] than in patients developing a metastatic disease [0.24(0.37)] during the follow-up period.

The T-NOR and the D-NOR significantly correlated with DNA ploidy (P = 0.0059 and P = 0.0199) (Table 2), in that aneuploid tumours had the higher Ag-NOR counts. The T-NOR values did not show any correlation with SPF (P = 0.5996). Crude (all deaths), cancer-related or recurrence-free survival were not related to Ag-NORs (Table 3).

The reproducibility of measurements (including intratumour and intraobserver variation) was assessed at random in 10 cases by counting the Ag-NORs twice at different times of measurement. The correlation coefficients for different types of NORs were: LRP; r = 0.76, A-NOR; r = 0.79, D-NOR; r = 0.66, T-NOR; r = 0.72.

DISCUSSION

More accurate prediction of tumour progression and patient survival would be of definite significance in the management of human breast cancer. Thus, a continuous search for the prognostic predictors is mandatory. In this respect, Ag-NOR counting has recently proved to be a promising new tool in a variety of malignant tumours [20–25, 29–36]. Prompted by these findings, the Ag-NOR technique was used to analyse a series of prospectively followed-up breast carcinomas in the present study.

Ag-NORs are located in acrocentric chromosomes, each chromosome having two of them. Not all Ag-NORs are visible in normal histological sections, however. Usually one or two may be present, free within the nucleus [33, 34]. By the end of G2 phase before mitosis, 20 Ag-NORs can be found in each normal nucleus [33]. The reports published on Ag-NOR counting so far have yielded contradictory results [20–25, 29, 32, 35]. Most studies have focused on counting the Ag-NORs in benign and malignant conditions [32, 35], but some attempts have been made to apply this method in predicting the prognosis of cancers as well [29]. With the considerable overlap of the Ag-NOR counts, it has become evident that this technique is not capable of separating malignant tumours from benign growths [32, 35]. Albeit a number of studies have shown some prognostic value for the Ag-NORs, the results involving cancer prediction have generally been disappointing [24, 29].

In breast cancer, a relationship between the Ag-NOR counts and malignancy has been reported [24, 35], although the differences in the Ag-NOR counts between different malignancy grades have not been dramatic [25, 35]. This could be confirmed also in the present series, where the high grade tumours showed higher Ag-NOR counts than the low grade lesions. Tumours with axillary lymph-node involvement (N+) at operation showed identical Ag-NOR counts to the tumours confined to the breast (N−). In contrast, tumours developing a metastatic disease (M+) during the follow-up had significantly higher Ag-NOR counts at examination. The Ag-NORs were separately studied also in premenopausal and postmenopausal women, but no differences were found when compared with the values in the whole material.

Some studies have not been able to establish any correlation between Ag-NOR counts and DNA ploidy [29, 31]. In the present series, however, aneuploid tumours had significantly higher Ag-NOR counts than the diploid ones. The results are in full agreement with those of Giri *et al.* [25]. Since in aneuploid cells, the number of chromosomes at any phase of the cell cycle is higher than in the diploid cells, higher Ag-NOR counts in aneuploid cells are to be expected. However, Ag-NORs represent active rRNA [30], and the proliferative activity of a given cell determines the Ag-NOR count. This might explain at least some of the discrepancies reported on the correlation between DNA content and the Ag-NOR count [25, 29, 31].

Differences in Ag-NOR counts in various cancers may also arise from the difficulty in evaluating the Ag-NOR particles, as well as from the liability of the method to staining artefacts due to different staining times [36]. In the present study, a generally accepted staining time [25] was used, and normal glandular or other epithelial cells served as internal controls usually showing one or two Ag-NOR particles per cell (Fig. 1). The counting of Ag-NOR particles was done with a simple method in which the Ag-NOR particles were grouped into four groups according to their size and shape. This method has been applied previously in bladder cancer [29], where good prognostic value was established for the Ag-NOR count. This scoring method has a good intraobserver reproducibility, and it is fully comparable to other methods in this respect [24, 25, 36].

The present series was carefully followed-up, thus representing an unselected group of patients with a long prospective

follow-up. The same material has been previously studied by means of histological grading [9], DNA flow cytometry [16], mitotic activity measurements [9] and MCA immunohistochemistry [19]. All these approaches have provided a number of prognostic factors with the predictive value superior to that obtained by the Ag-NOR counting in the present study [9, 16, 19]. On the basis of the present results, it seems justified to conclude that the Ag-NOR counting does not add any significant information in prediction of the breast cancer. The studies focused on discriminating malignancy from benign conditions seem more promising [32], but the present study cannot give any eludication on this issue. We feel, however, that the enumeration of Ag-NORs is a more powerful prognostic parameter in slowly progressing tumours than in usually aggressively behaving ductal breast carcinoma. From the same reasons histological grading is of rather limited prognostic value in predicting clinical outcome in individual cases of breast cancer.

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