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Nucleolar grading of breast cancer

Comparative studies on frequency and localization of nucleoli and histology, stage, hormonal receptor status and lectin histochemistry

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Summary. The localization and number of nucleoli of tumour nuclei were examined after conventional histological typing of breast carcinomas (WHO classification), staging, biochemical receptor status, and lectin binding site histochemistry. With increasing histological atypia and grade of malignancy, the frequency of nucleoli increased significantly and their location shifted from the center to the nuclear periphery. These changes correlated with an increase in indices of mitosis and cell loss. The nucleolar size correlated with the increase in the grade of malignancy. Hormone receptor and lectin binding site positive carcinomas were characterized by a low nucleolar frequency and by small nucleoli in central position, whereas carcinomas with high grades of malignancy and negative hormone receptor and lectin binding site status showed large, often multiple nucleoli in eccentrical position at a high frequency. The studies have demonstrated that nucleolar status is an easily practicable histological/cytological method for use in the assessment of prognosis of carcinomas of the breast.

Key words: Breast cancer – Nucleolar status – Grading – Hormone receptor

Introduction

The presence or absence of steroid hormone receptors in breast cancers has proved to be an important factor in therapy and prognostic evaluation. In addition, proliferation rate and grade of malignancy have been shown to be prognostic indicators. In breast cancer a separation may be made between nuclear and histological gradings, the latter usually performed in accordance to the proposals of Bloom and Richardson (1957) and the former on the methods of Black and Asire (1969).

Malignancy may be assessed numerically if different histological degrees of differentiation and cytological degrees of atypia are given in a grading score (Müller et al. 1980). The nucleolar character is also considered in some systems of cytomorphological grading (Helpap 1988a). In combination with hormone receptor status, such examinations have been performed on biopsy smears from breast carcinoma (Schenck et al. 1986; Weintraub et al. 1987) but the exact number and localization of nucleoli have not been given much attention, though they seem to be an important feature in determination of the grades of malignancy of tumours. We have demonstrated their importance on tumour precursors and manifest carcinomas of the prostate (Helpap 1988a). This report compares morphology, hormone receptor status, and the demonstration of peanut lectin in breast carcinomas in a combined histological/cytological grading with special regard to nucleolar pathology.

Material and methods

102 breast carcinomas were examined histopathologically after primary frozen section. In the course of the frozen sections, tissue samples were frozen in order to achieve a biochemical receptor determination. The determination of steroid hormone receptors was performed biochemically on the tumour homogenisate by use of the dextran coated charcoal (DCC) method. Special attention was payed to removing all nontumorous tissue. The normal limit for estrogen receptor (ER) contents was fixed at 10 fmol/mg tissue protein, for progesteron receptor (PR) contents at 20 fmol/mg tissue protein. Results below these limits were considered to be receptor negative (Klinga et al. 1982). On paraplast embedded material, peanut lectin was determined histochemically by use of the direct ABC-method.

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Table 1. Correlation of histology with mitosis, nucleolar frequency and localization in breast carcinomas

Histology	*n*	Apoptosis %	Mitosis %	Nucleolar				Localization		Nucleoli/nuclei ratio	
				frequency %	number			central	peripheral	<1:8	>1:8
					N1	N2	N3				
CLIS	6	–	–	3.8 ±3.5	97.6 ±5.8	2.4 ±2.2	–	90.5 ±11.0	9.5 ±8.5	100.0	–
Mucinous Ca.	3	–	–	10.6 ±6.2	100.0	–	–	89.3 ±18.5	10.7 ±9.2	100.0	–
Intraductal Ca.	12	0.2	0.3	6.4** ±5.1	87.9 ±27.7	12.1 ±11.2	–	79.7** ±23.3	20.3** ±19.9	91.7	8.3
Medullary Ca.	4	1.5** ±0.4	1.9** ±0.9	16.8 ±15.9	93.0 ±8.7	6.7 ±5.6	–	42.0** ±17.6	58.0** ±17.6	33.3	66.7
Invasive lobular Ca.	20	0.5** ±0.3	0.3** ±0.3	29.4 ±25.3	91.2 ±13.3	8.3 ±6.1	0.5** ±0.5	63.2* ±21.3	36.8* ±17.0	25.0	75.0
Invasive ductal Ca.	57	1.3** ±0.7	1.4** ±0.7	38.2** ±24.7	85.4 ±23.9	11.3 ±8.0	3.3** ±2.3	48.6** ±20.1	51.4** ±19.7	12.3	87.7

Statistically significant: * $p<0.05$, ** $p<0.005$ (Student's *t*-test)

Deparaffinated tissue sections were incubated with biotin conjugated PNA after pretreatment with methanolic H_2O_2 solution. Some of the sections had been previously exposed to neuraminidase in order to reveal masked binding sites. Biotin was labelled with streptavidin conjugated peroxidase, and the sections were stained using the substrate DAB. The sections were counterstained with haemalaun and treated in the usual manner. Upon evaluation, the share of stained tumour cells was registered as low (less than 30%) (+), moderate (30 to 60%) (++) and high (more than 60%) (+++). By measuring of the largest tumour diameter and isolated preparation of lymph nodes from different localizations, the T and N status was determined (Hermanek and Sobin 1987).

The tumours were classified according to the WHO-classification after formalin fixation, paraplast embedding, and staining with haematoxylin-eosin and PAS, also using connective tissue stains (van Gieson, sirius red, elastica, and Goldner) (Azzopardi 1982; WHO 1981).

The grading of malignancy was performed according to Bloom and Richardson (1957). Besides the histological pattern special attention was payed to the frequency of nucleoli, the occurrence of one, two, or more nucleoli per nucleus, and the localization of nucleoli in central and eccentric positions. The size of the nucleoli was related to the nuclear size and divided in groups <1:8 and >1:8. The rate of cell proliferation (%index of mitosis) and the defined of cell loss (%index of apoptosis) were measured. Apoptosis is as a lysosomal phagocytic process of intracytoplasmic remnants of condensed nuclear and cytoplasmic particles after nuclear pyknosis or karyorrhexis (Helpap 1989; Wyllie 1987).

On average, 1000 nucleoli were counted per case of carcinoma using a graticule in the eyepiece of the microscope. The nucleolar frequency, size and localization were correlated to the histological pattern, the receptor and lectin status, and the grading. The data were analyzed by means of Student's unpaired *t*-test. *p* values <0.05 were considered significant.

Results

Among the 102 breast carcinomas, we found 57 invasive ductal carcinomas, 20 invasive lobular, 4 medullary, 12 intraductal, 3 mucinous carcinomas, and 6 lobular carcinomata in situ. In carcinomata in situ and mucinous carcinomas, apoptoses and mitoses were not encountered.

Intraductal carcinomas and invasive lobular carcinomas showed very low percentages of cell loss rate (apoptosis rate) and mitosis rate. The highest percentages were registered in medullary and invasive ductal carcinomas (Table 1).

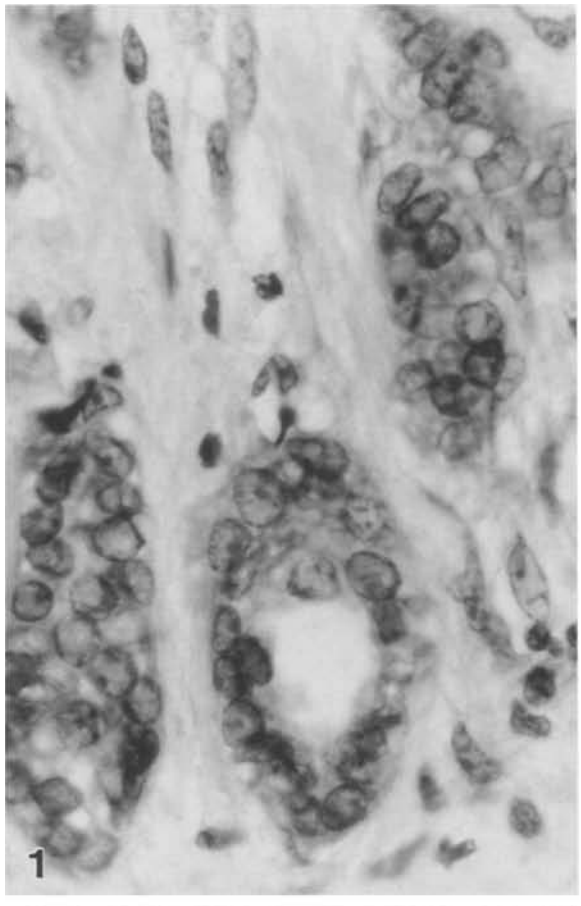
The percentage of nuclei with nucleoli was lowest in lobular carcinoma in situ and intraductal carcinoma. Invasive lobular and invasive ductal carcinomas yielded the highest values at up to 38.2% (Table 1). The number of nucleoli per nucle-

Fig. 1. Differentiated invasive ductal carcinoma with very low nucleolar frequency. H.E. 504×

Fig. 2. Large nucleoli in invasive lobular carcinoma. H.E. 504×

Fig. 3. Medullary carcinoma with high nucleolar frequency but single nucleoli in eccentrical localization. H.E. 504×

Fig. 4. Invasive ductal carcinoma with high nucleolar frequency, multiple nucleoli, and eccentrical localization. H.E. 504×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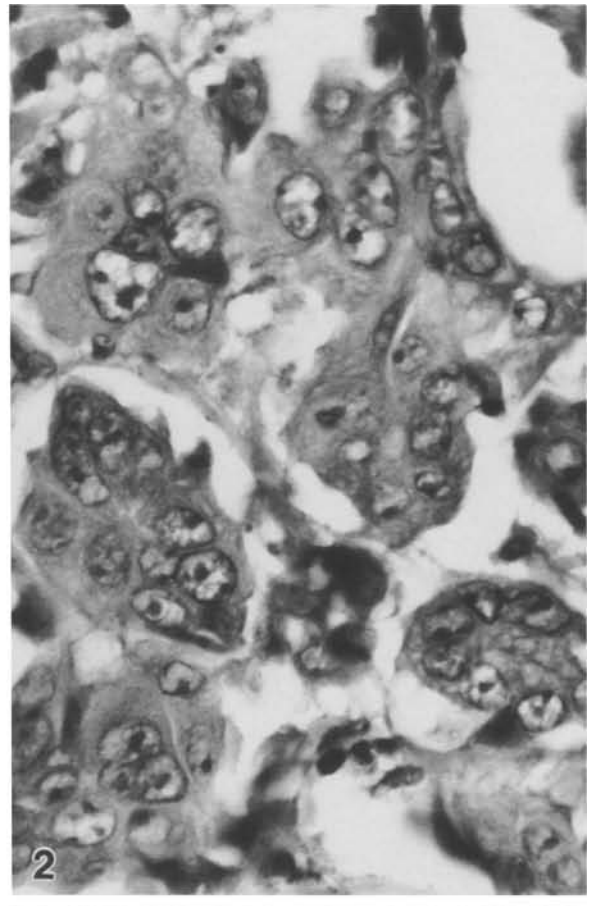
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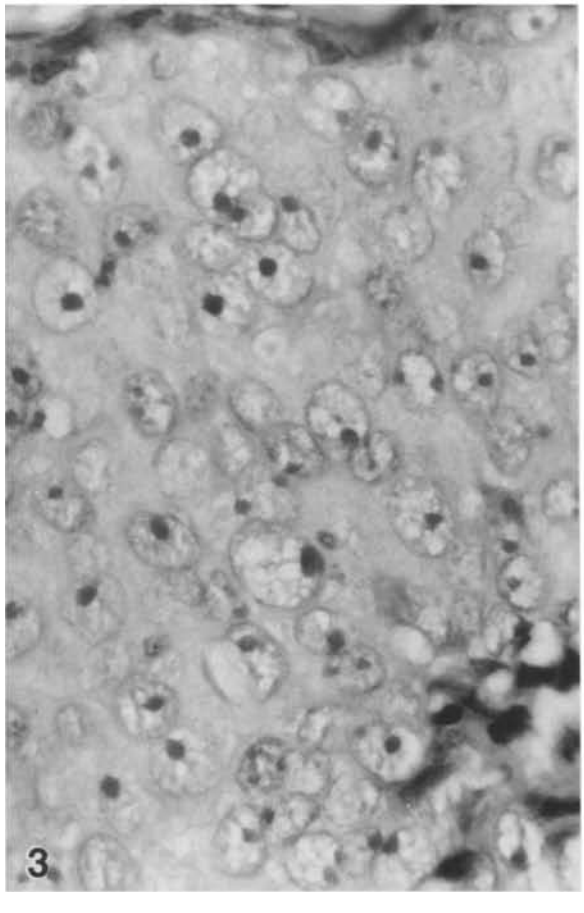
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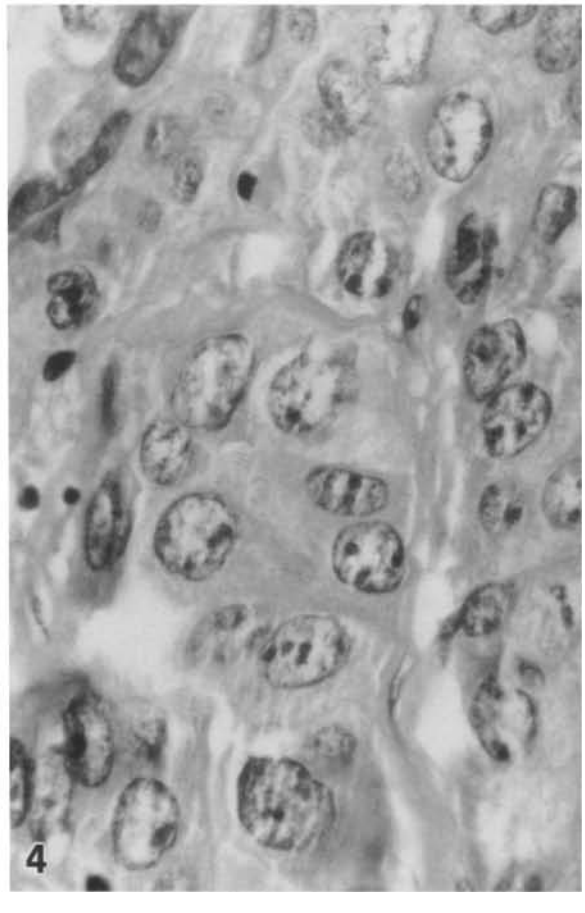
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Table 2. Correlation of grading with mitosis, nucleolar frequency and localization in breast carcinomas

Grading	*n*	Apoptosis %	Mitosis %	Nucleolar				Localization		Nucleoli/nuclei ratio	
				frequency %	number			central	peripheral	<1:8	>1:8
					N1	N2	N3				
G I	16	–	0.01	4.4** ±4.2	100.0*	–	–	80.7* ±20.1	19.3* ±11.7	100.0	–
G II	40	0.6** ±0.5	1.1* ±0.7	15.3** ±12.4	94.9* ±15.4	5.0** ±3.6	0.1	65.1** ±22.2	34.9** ±14.7	42.5	57.5
G III	46	0.9** ±0.4	1.5* ±0.7	56.9** ±9.9	86.3* ±16.4	11.8** ±6.4	1.9 ±1.4	50.1** ±21.7	49.9** ±27.3	–	100.0

Statistically significant: * $p<0.05$, ** $p<0.005$ (Student's *t*-test)

Table 3. Correlation of tumour stage (pT) and nucleolar status

Stage pT	*n*	Apoptosis %	Mitosis %	Nucleolar frequency and number				Localization	
				%	N1	N2	N3	central	peripheral
1	38	0.3** ±0.3	0.3** ±0.3	17.8** ±16.5	92.7 ±19.9	5.6** ±2.9	1.7 ±1.5	62.2 ±25.1	37.8 ±24.7
2	52	0.6** ±0.5	0.6** ±0.5	34.0** ±28.0	89.1 ±17.2	9.4** ±8.3	1.5 ±1.4	61.4 ±24.4	38.6 ±24.3
3/4	12	0.3* ±0.3	0.5 ±0.5	32.0 ±5.7	91.1 ±8.9	8.9 ±8.8	–	56.7 ±23.4	43.3 ±26.6

Statistically significant: * $p<0.05$; ** $p<0.005$ (Student's *t*-test)

us increased in a similar pattern. In carcinomas, not only 2, but in 3.3% of cases 3 or more enlarged nucleoli could be counted per nucleus (Table 1). There was similar behavior in nucleolar localization. Lobular carcinomata in situ, mucinous carcinomas and intraductal forms had the highest percentages of nucleoli in central positions, whereas invasive lobular and ductal as well as medullary carcinomas showed nucleoli in eccentric positions in 36 to 58% (Figs. 1–4).

In 32% of 102 breast cancers examined, the tumour cells showed small nucleoli, in 68% large nucleoli (ratio nucleolus/nucleus <1:8, respectively, >1:8). Lobular carcinomata in situ yielded the highest percentages of small nucleoli, followed by the mucinous carcinomas. Medullary, invasive lobular and ductal carcinomas were characterized by large nucleoli. Breast cancers grade of malignancy G I showed small nucleoli exclusively, G III carcinomas large nucleoli only. In G II carcinomas, the cases with large nucleoli predominated. In cases of carcinomas with small nucleoli low rates of apoptosis and mitosis were found as well as a low nucleolar frequency with mainly 1 nucleolus per nucleus in predominantly central position. In contrast, carcinomas with large nucleoli were characterized by a sixfold higher frequency of nucleoli and accordingly increased rates of apoptosis and mitosis, by occurrence of 2 and more nucleoli per nucleus, and by almost 50% peripheral localization of nucleoli (Tables 1, 2; Figs. 1–4).

With increasing grade of malignancy, the frequency of apoptosis and mitosis increased distinctly ($p<0.005$). The nucleolar frequency increased from 4.4% in G I-carcinomas to 56.9% in G III-carcinomas. The poorly differentiated types of carcinomas showed more than 1 nucleolus per nucleus in up to 11.8%. The nucleoli shifted towards the nuclear periphery in up to 50% of the cases in G III carcinomas. The differences were highly significant, $p<0.005$ (Table 2; Figs. 2–4).

The increase in size of carcinomas from pT1 to pT2 was accompanied by an increase in frequency of nucleoli from 17.8% to 34% ($p<0.005$). There was no remarkable change in the number of nucleoli per nucleus and in nucleolar localization. In 90%, a single nucleolus, and in 6–9%, 2 nucleoli per nucleus were found ($p<0.005$). The central position predominated in 60% ($p<0.005$). Compared to stage pT2, no changes could be detected in stages pT3 and pT4 with regard to the frequency and localization of nucleoli (Table 3).

Table 4. Correlation of hormone receptor status and lectin binding proof to nucleolar frequency, localization, apoptosis, mitosis and nucleolar size

		($n=69$)			($n=10$)		
		ER +	PR +	lectin +++	ER −	PR −	lectin −
Nucleolar frequency	%	24.3*	±24.2		47.6		±28.0*
N 1	%	92.6	±18.8		87.8		±14.8
N 2	%	6.5	±2.6		9.8		±8.8
N 3	%	0.9*	±0.9		2.4*		±2.1
Localization central	%	64.4**	±24.3		46.9**		±16.7
Peripheral	%	35.6**	±23.9		53.1**		±16.7
Apoptosis	%	0.5	±0.4		0.6		±0.6
Mitosis	%	0.4*	±0.3		0.9*		±0.6
Nucleolar prominence							
>1:8	%	64.5			100.0		
<1:8	%	35.5			0		

Statistically significant: * $p<0.05$, ** $p<0.005$ (Student's t-test)

In cases with positive estrogen (ER) and progesterone (PR) receptor tests as well as positive demonstration of lectin binding sites, the frequency of nucleoli was lowest among the breast carcinomas. In 65%, the nuclei showed one large nucleolus. The central nucleolar position prevailed with 64% (Table 4). With the positive estrogen receptor test, negative progesterone receptor, but positive demonstration of lectin binding sites, a higher nucleolar frequency with large nucleoli was found. The position of the nucleoli was in half central, in half peripheral. With negative receptor status but strongly positive lectin demonstration, the nucleolar frequency was slightly lower. However, there was an increased occurrence of 2 nucleoli per nucleus. Almost half of the nucleoli had taken an eccentrical position with the nucleus. In 2/3 of the cases, there were large nucleoli. With negative receptor status and lacking lectin proof, the eccentric nucleolar position predominated; the highest nucleolar frequency was found with increased occurrence of 2 or 3 nucleoli per nucleus and exclusively large nucleoli ($p<0.05/0.005$) (Table 4).

Discussion

Numerous examinations have shown that non-invasive carcinomas have highly positive values for estrogen and progesterone receptors. This is also true for mucinous carcinomas, whereas the invasive ductal and lobular carcinomas yield moderately to faintly positive values and a high share of receptor negative cases. Of medullary carcinomas, only a small number are receptor positive (Parl et al. 1980, 1984; Lesser et al. 1981; Dizerenz et al. 1985).

A high percentage of these biochemically receptor negative cases, however, give positive reactions for lectin binding sites; thus the rate of ER-, PR-, and lectin negative carcinomas will finally arrive at 4 to 8% (Helpap 1988b). The susceptibility to therapy and prognosis of breast cancers, however, is not only influenced by hormone receptor and lectin binding site status, but also by histological and cytological variables.

In addition to the morphological classification, the determination of the grade of malignancy of a breast cancer comprises the proliferative behavior. The various proliferative kinetic examinations have shown that the share of estrogen and progesterone receptor positive cases decreases and the share of negative cases increases with increasing proliferative tendency, shown by increase of autoradiographic labelling index (thymidine labelling index), with increasing percentage of S-phase cells, increase of grades of aneuploidy, increase in Ki 67 and epidermal growth factor expressing cells and in cell loss rate, measured by apoptosis index and tumour necrosis factor (Barnard et al. 1987; Feichter et al. 1985; Franklin et al. 1987; Gerdes et al. 1986; Lee et al. 1985; Lelle et al. 1987; Meyer et al. 1977, 1978, 1979, 1980, 1983; Möller et al. 1989; Moran et al. 1984; Müller-Holzner et al. 1983; Rank et al. 1987; Remvikos et al. 1988; Silvestrini et al. 1979; Stiens and Klotz 1985; Strauss et al. 1982; Tubiana et al. 1984).

Usually, invasive ductal and lobular and medullary carcinomas with high proliferative rates are hormone receptor negative (McGurrin et al. 1987; Reiner et al. 1987, 1988; Wrba et al. 1987, 1988a, b). It can therefore be concluded that carcinomas of high malignancy are receptor negative in a higher percentage than well differentiated G I-carcinomas (Dizerenz et al. 1985; Reiner et al. 1985).

Besides the nucleolar pattern, the argyrophilia of the nucleolar organizer regions proteins acts as a marker of ribosomal RNA and possibly of its level of transcription. This may provide useful information about the structure of the nucleolus and nucleolar activity in hyperplastic and neoplastic conditions (Walker 1988). With the argyrophil technique to identify nucleolar organizer regions (Ag-NORs) it was found that the total number of Ag-NORs in malignant breast lesions significantly exceeded those of normal breast and benign lesions (Smith and Crocker 1988; Bondi et al. 1989). Similar results were found on normal, hyperplastic benign and malignant neoplastic condi-

tions of lymph nodes (Crocker and Nar 1987), melanocytic lesions (Crocker and Skilbeck 1987), and cutaneous tumours (Egan and Crocker 1988). Nucleolar organizer staining and Ki 67 immunostaining in non-Hodgkin's lymphoma suggest that the mean number of nucleolar organizer regions may reflect the cellular kinetics of a tumour and that the mean nucleolar organizer region score is related to the histological grade of non-Hodgkin's lymphomas (Hall et al. 1988). The exact proportion of nucleolar frequency, number, size and localization of nucleoli to the argyrophilia staining results (AgNOR technique) for nucleolar organizer regions of the different breast carcinomas and precursors as well as atypical adenosis or proliferative atypical mastopathia must be investigated in future. At the present time, however, there is no good evidence that the method is of value in the diagnostically important areas of borderline lesions or in prognosis (Walker 1988).

The cytomorphological grading has also established that with increasing grade of nuclear atypia, the share of receptor negative carcinomas increases (Fisher et al. 1987). Thus it has been shown that the share of receptor positive carcinomas decreases from 97% to 37% with increasing grade of atypia; the highest grades of atypia are found in receptor negative carcinomas (Schenck et al. 1985). This statement is based on the cytological analysis of biopsy smears from breast carcinomas; the criteria for cellular atypia were divided in 8 groups which were discriminated by absence or presence of small or large nuclei with or without enlarged, solitary or multiple, nucleoli (Mattfeld et al. 1985; Schenck et al. 1986). The relationship of nuclear size and estrogen receptor positivity in morphometric studies has also shown that carcinomas with small nuclei exhibit higher ER concentrations than carcinomas with large nuclei (Guazzi et al. 1985; Schenck et al. 1986).

Our own examinations on frequency and localization of nucleoli have emphasized the importance of this easily detectable cytological variable. This becomes evident through an increase in solitary nucleoli and occurrence of multiple nucleoli as well as through the increasingly eccentric position within the nucleus with increasing grade of malignancy and increase in dedifferentiated histological structures and invasive tendency of the carcinomas. The distribution pattern and the enlargement of the nucleoli supports the analysis. The highly malignant invasive carcinomas (G II and G III) are characterized by large solitary or multiple nucleoli whereas in situ growing carcinomas and mucinous carcinomas with low grade of malignancy (G I) show small solitary nucleoli. The medullary carcinomas not infrequently show small nucleoli. This correlates with the other cytological findings and stresses the heterogeneity of different cell clones; thus the biological behavior of medullary carcinomas must be separated in a favorable sense from the invasive ductal carcinomas. The correlation between the increase in nucleolar frequency, eccentric nucleolar position, occurrence of multiple nucleoli per nucleus and increasing grade of proliferative activity of the carcinomas was demonstrated very exactly on prostatic carcinomas by cytological cell kinetic and autoradiographical analysis (Helpap 1988a, 1989).

The nucleolar status of the breast carcinomas examined was also correlated with hormonal receptor and lectin binding site status. There was a low nucleolar frequency in hormonal receptor and lectin binding site positive carcinomas with mainly centrally localized nucleoli, whereas the hormonal receptor and lectin binding site negative carcinomas were characterized by a distinctly higher nucleolar frequency with increasing eccentric position of the nucleoli and occurrence of multiple nucleoli per nucleus. Thus, these analyses are in accordance with the correlation between degree of nuclear atypia and receptor status of breast carcinomas in the literature (Guazzi et al. 1985; Mattfeld et al. 1985; Schenck et al. 1985, 1986; Fisher et al. 1987).

In contrast to the clear correlation between histology, grading, receptor/lectin binding status, and nucleolar pathology, the associations of nucleolar frequency and localization and the increase in size of the carcinomas is not unequivocal. There is an increase in nucleolar frequency only from pT1 to pT2 tumours. The increase from pT2 to pT3/4 tumours is not significant. There is no significant difference in the nucleolar localization between the different tumour extension stages.

On the basis of the correlation between nucleolar pattern and histological and cytological grading we may expand the grading system of Bloom and Richardson (1957) by the following score of histological, nuclear and nucleolar findings to 1 point = mild changes, 2 points = moderate changes, 3 points = marked changes. The score consists of the sum of the histological and cell kinetic points and the points given to the amount of nuclear and nucleolar atypia. Three grades of malignancy are then reduced from the results (Table 5). It must be stressed that carcinoma lobulare in situ, intraductal carcinoma, and highly differentiated glandular and mucinous carcinomas are characterized by very small nucleoli with low frequency and singular

Table 5. Histological and cytological grading of carcinomas of the breast. Modified after Bloom and Richardson (1957)

(1)	Histological pattern	Score
	Well differentiated mature tubuli	1
	Moderately differentiated to immature tubuli	2
	Little to no differentiation	3
(2)	Nuclear Pattern	
	Isomorphia (equal nuclear size classes)	1
	Variability in size and shape (different nuclear size classes)	2
	Bizarre shapes, giant nuclei	3
(3)	Nucleolar pattern	
	Small solitary nucleoli in central location	1
	Large solitary nucleoli in central and peripheral location	2
	Large (prominent) multiple nucleoli eccentrically located	3
(4)	Rate of mitosis and apoptosis per high power field	
	Scattered mitosis and apoptosis	1
	2–3 Mitoses and apoptotic bodies	2
	More than 3 mitoses or apoptotic bodies	3

Score in total	Grade of malignancy
4	G I
5–8	G II
9–12	G III

occurrence in a predominant central localization, that highly malignant invasive ductal and lobular carcinomas as well some medullary carcinomas are characterized by large (prominent) solitary and multiple nucleoli with mainly eccentrical localization, that breast cancers with high rates of mitosis and DNA synthesis correlate with high nucleolar frequency, multiple nucleoli per nucleus and eccentrical nucleolar localization, and that therefore, low nucleolar frequency and central localization are observed in carcinomas with positive hormone receptors and positive lectin binding sites. However, high nucleolar frequencies, multiple nucleoli, and eccentrical positions can be found in carcinomas with negative hormone receptor and lectin binding site tests.

From the demonstrated correlations between histology, cytology, cell kinetics, immunohistochemistry, and hormone receptor/lectin binding site status it is clear that this easily detectable cytological finding presents is a practicable means of determining grades of malignancy and prognosis in breast cancer.

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