Prognostic Factors and Proliferative Activities in Breast Cancer. A Comparative Study between Recurrent and Non-Recurrent Cases of Human Breast Carcinomas

Yoshiaki Imamura, Makoto Ishida, Kohjiro Horita and Masaru Fukuda

Department of Pathology, Fukui Medical School, Matsuoka, Yoshida-gun, Fukui, 910-11

Received for publication February 1, 1997

Twenty-one recurrent and twenty-six non-recurrent cases of breast carcinoma were compared with respect to prognostic factors and proliferative activities. The mean number of lymph nodes involved (lymph node score) and tumor stage in recurrent and non-recurrent cases were $10.0 \pm 14.5/2.05 \pm 0.59$ and $0.96 \pm 2.75/1.42 \pm 0.50$, respectively. Expression of p53/c-erbB-2 was demonstrated immunohistochemically in 28.6/52.4% of recurrent cases, and in 15.4/34.6% of non-recurrent cases, respectively. PCNA indices in the two groups

were 47.8 ± 14.2 and 39.5 ± 18.2 , respectively. Fluorescent nucleolar organizer regions (FINORs) scores determined by confocal laser scanning microscope in the two groups were 3.03 ± 0.78 and 2.32 ± 0.54 , respectively. There were significant differences between the two groups in lymph node score, tumor stage, and FINORs score, but not in the expression of p53 or c-erbB-2 or in PCNA indices. These findings indicate that lymph node score, tumor stage, and FINORs score may be useful as prognostic factors in patients with breast carcinoma.

Key words: Breast carcinoma, Prognosis, p53, PCNA, Fluorescent nucleolar organizer regions

I. Introduction

The clinical course of human breast carcinoma is often unpredictable. Conventional clinical staging and histopathological grading systems do not always predict the course of individual tumor development satisfactorily [19]. Additional variables are needed to give supplementary information that can lead to a better understanding of the tumor biology of breast carcinoma and give a cell biological basis for the choice of adequate treatment.

Recently, many alterations in DNA were demonstrated in human breast carcinoma, and their clinical and pathological implications were extensively examined. Among these alterations, amplification of the c-erbB-2 proto-oncogene [1, 4] and mutation of the p53 tumor suppressor gene [2, 31] have been demonstrated frequently in human breast carcinoma tissues. Moreover, overexpressions of c-erbB-2 [20, 33, 34] and p53 [9, 15, 28] oncoproteins were suggested to be associated with poor

prognosis of breast carcinoma. However, other investigations indicate the lack of evidence for prognostic significance of overexpressions of c-erbB-2 [35] and p53 [5, 6] oncoproteins.

The proliferative capacity of a malignant neoplasm is an important adjunct to histological grading and may be an indicator of treatment response and relapse in many tumors [29, 30]. Good correlation has been found between high cell proliferation rates measured by flow-cytometry and poor short-time survival in primary breast carcinomas [10]. Cell kinetics has been determined by thymidine-incorporation, bromodeoxyuridine labeling index, flow-cytometry, or counting mitotic figures. Recently, monoclonal antibodies against the 36 kDa, S-phase-associated, nuclear protein: proliferating cell nuclear antigens (PCNA) have been developed for use in immunohistochemistry [32]. Several studies have shown that some of these antibodies, especially that derived from a clone designated PC10, are suitable for immunohistochemical investigations in conventionally fixed and processed tissue [24, 32]. In breast tissue or carcinoma, PCNA immunoreactivity has been shown to correlate with thymidine labeling index [3], DNA flow-cytometry [21], and Ki-67 immunoreactivity [7].

The enumeration of nucleolar organizer regions

Selected by "The 10th International Congress of Histochemistry and Cytochemistry" held in Kyoto, Japan on Aug. 18–23, 1996.

Correspondence to: K. Horita, M.D., Department of Pathology, Fukui Medical School, Matsuoka, Yoshida-gun, Fukui, 910-11, Japan.

(NORs) in neoplastic cell nuclei is also a relatively new method for studying the cell kinetics [8]. They are closely associated with argyrophilic proteins and can be visualized as AgNORs in paraffin sections by a simple silver stain technique [23]. These proteins are possibly related to RNA polymerase I, or other proteins such as C₂₃ and B₂₃ [12]. The number of AgNORs in the interphase tumor cell nuclei is supposed to be related to ribosomal gene activity, and therefore to cell proliferation with possible prognostic implications. We developed a new method of fluorescent staining of nucleolar organizer regions (FINORs) [14]. In this method, aluminum ammonium sulfate was used instead of silver as the cationic metal ion for binding with NORs-associated proteins, and fluorescent morin was successively bound to aluminum by chelating with modification of the method developed by Malinin [16]. We examined human benign and malignant tumors by three-dimensional analysis of the fluorescent images of FINORs under confocal laser scanning microscope (CLSM) and found a remarkable increase of FINORs not only in number, but also in volume, with bizarre configuration in the process of tumor progression [14].

In the present study, we compared prognostic factors (the number of lymph nodes involved: lymph node score, tumor stage, and the expression of p53 and c-erbB-2 oncoproteins) and proliferative activities (PCNA index and the number of FINORs: FINORs score) between twenty-one recurrent and twenty-six non-recurrent cases of breast carcinoma. Our purpose is to examine whether or not these factors would provide useful information concerning prognosis in patients with breast carcinoma.

II. Materials and Methods

Materials

From a series of one hundred forty-five patients presenting with primary breast carcinomas who had undergone radical or modified radical mastectomy with lymph node dissection at the Department of Surgery, Fukui Medical School among 1983 and 1990, twenty-one recurrent cases (age range 28–81 years: median 53.2±13.9 years) within five years and twenty-six non-recurrent cases (age range 27–82 years: median 56.7±14.1 years) more than five years were used in this study (Tables 1 and 2). The mean follow up period of non-recurrent cases was 95.2 months (range 61–128 months). Twenty normal breast tissues adjacent to carcinomas were used for control.

Pieces of tumor and axillary lymph nodes were fixed in 10% neutrally buffered formalin, and embedded in paraffin. Sections 4 µm thick were stained with hematoxylin and eosin, and were examined histopathologically. All samples were classified according to the World Health Organization (WHO) histological typing of breast tumors. Two clinico-pathological parameters (lymph node status and tumor stage), considered as being of prognostic significance, were also evaluated. T staging was

performed according to the American Joint Committee on Cancer Criteria Schema.

Immunohistochemistry

For immunohistochemistry, 5 µm sections from primary tumors were cut and dewaxed. The sections were placed in 0.3% hydrogen peroxide in methanol solution to block endogenous peroxidase activity. After washing in PBS, the sections were incubated with 10% normal rabbit serum for 10 min. Sections were washed in PBS and then coated with anti-p53 oncoprotein monoclonal antiserum (Ab-6, Oncogene Science, Inc., New York, USA, 1/50 in PBS), anti-c-erbB-2 oncoprotein monoclonal antiserum (NCL-CB11, Novocastra Laboratories Ltd., Newcastle, UK, 1/50 in PBS), or anti-PCNA monoclonal antiserum (PC10, Novocastra Laboratories Ltd., UK, 1/50 in PBS) and left at room temperature overnight. Sections were washed in PBS and incubated with secondary antiserum (biotinylated rabbit anti-mouse immunoglobulin [Nichirei Ltd., Tokyo, Japan]) at room temperature for one hr and then rewashed in PBS. Treatment with avidin-biotin-peroxidase complex (avidin-peroxidase-conjugated streptavidin [Nichirei, Japan]) followed at room temperature for one hr. Immunoreaction products were visualized by 0.02% 3,3'-diaminobenzidine tetrahydrochloride (Dojin Ltd., Kumamoto, Japan) with 0.05% hydrogen peroxide, and all sections were counterstained with hematoxylin. Negative control sections were incubated with non-immunized normal rabbit IgG at room temperature overnight.

In each specimen, over 500 cells were counted in random field. Staining of the nucleus was considered to be positive expression of p53 and PCNA, and staining of the cytoplasmic membrane was estimated to be a positive expression of c-erbB-2. Staining intensity of p53 and c-erbB-2 was expressed as negative (–), less than 10% of positive cells (±), from 10 to 50% of positive cells (+), and more than 50% of positive cells (++). Staining scores of p53 and c-erbB-2 were determined as 0 is –; 1, ±; 2, +; and 3, ++. The number of PCNA-positive tumor cells divided by the total number of tumor cells counted provided a PCNA index for each tumor.

FINORs staining

10 µm sections were prepared. The de-paraffinized sections were washed with ion-free distilled water. They were reacted in 5% aluminum ammonium sulfate (Nacalai Tesque, Inc., Kyoto, Japan) dissolved in ion-free distilled water at room temperature for one hr. After thorough washing with ion-free distilled water, the sections were reacted with the metal-chelating compound, 0.05% morin (3,5,7,2',4'-pentane-hydroxyflavonol, Nacalai Tesque, Inc., Japan) dissolved in ion-free distilled water at room temperature for 30 min in the dark, and washed again with ion-free distilled water. They were mounted with 1% para-phenylenediamine (Nacalai Tesque, Inc., Japan) dissolved in PBS to prevent the fluorescence bleaching.

Observation of FINORs by confocal laser scanning microscope (CLSM)

The confocal laser scanning microscope (LSM-GB200, Olympus, Tokyo, Japan) was equipped with an inverted fluorescence microscope (IMT-2-21-LSM, Olympus, Japan) and an air-cooled argon laser. For the excitation of morin fluorescence, the 488 nm laser line was used. A 535 nm band pass filter (BP535, Olympus, Japan) was used in the fluorescence detection path. The fluorescence was collected using an oil-immersion objective lens, Olympus Splan-Apo $\times 100$ (NA=1.4) with the zoom ratio, $\times 8.0$ and scanning speed of 40 sec. Optical sections of FINORs were obtained by moving up the sample stage using a piezo electric translator at intervals of 1 μ m. Nine images of each nucleus were reconstructed by host computer (HP Vectra386/33U, YHP, Tokyo, Japan). The number of FINORs (FINORs score) was counted by using the reconstructed stereo images. The mean number of FINORs in one hundred cell nuclei considered as FINORs score for each case.

Statistical analysis

The t test was applied to compare lymph node scores, PCNA indices, and FINORs scores between recurrent and non-recurrent cases. Differences of T stages, p53 scores, and c-erbB-2 scores between the two groups were tested by using the chi-square test. A p value of less than 5% was considered significant.

III. Results

Clinico-pathological findings

All forty-seven infiltrating carcinomas in this study were ductal carcinomas.

Among the twenty-one recurrent cases, ten cases involved more than two different organs and eight out of them showed cancer death. On the other hand, the other eleven recurrent cases involved single organ and only two cases showed cancer death (Table 1). No cancer death was detected in all twenty-six non-recurrent cases (Table 2).

The number of lymph nodes involved (lymph node score) of each case is shown in Tables 3 and 4. The mean lymph node scores in recurrent and non-recurrent cases were 10.0 ± 14.5 and 0.96 ± 2.75 , respectively (Table 5). There were significant differences between the two groups in lymph node score by t test ($p < 0.01$).

Tumor stage of each case is shown in Tables 3 and 4. The mean tumor stages in recurrent and non-recurrent cases were 2.05 ± 0.59 and 1.42 ± 0.50 , respectively (Table 5). There were also significant differences between the two groups in tumor stage by chi-square test ($p < 0.05$).

Immunohistochemistry

Overall, of the forty-seven tumors evaluated, ten (21.3%) demonstrated variable levels of p53 immunoreactivity in neoplastic cell nuclei (Fig. 1-A, B), whereas thirty-seven (78.7%) did not express any immuno-

Table 1. Clinical course of recurrent case

Case No.	Age (year)	Recurrent sites	Follow up period (month)	Prognosis
1	54	multiple	16	died
2	67	multiple	49	died
3	52	multiple	65	died
4	71	multiple	11	died
5	28	multiple	11	died
6	66	multiple	32	died
7	44	multiple	22	died
8	30	multiple	79	alive
9	81	local	85	died*
10	60	lymph node	133	alive
11	45	bone	103	alive
12	60	lymph node	88	alive
13	38	bone	75	alive
14	51	local	86	alive
15	44	skin	58	died
16	74	skin	74	alive
17	57	lung	56	died
18	48	skin	62	alive
19	41	multiple	15	died
20	49	multiple	54	alive
21	57	lymph node	38	alive

*Died of the other cause

Table 2. Clinical course of non-recurrent case

Case No.	Age (year)	Recurrent sites	Follow up period (month)	Prognosis
22	35	—	128	alive
23	51	—	117	alive
24	51	—	113	alive
25	57	—	112	alive
26	42	—	111	alive
27	61	—	111	alive
28	59	—	106	alive
29	73	—	105	alive
30	64	—	104	alive
31	69	—	103	alive
32	55	—	102	alive
33	74	—	99	alive
34	63	—	99	alive
35	82	—	98	alive
36	68	—	97	alive
37	37	—	96	alive
38	54	—	92	alive
39	63	—	87	alive
40	76	—	85	alive
41	47	—	84	alive
42	58	—	81	alive
43	40	—	79	alive
44	58	—	74	alive
45	39	—	68	alive
46	27	—	63	alive
47	72	—	61	alive

Table 3. *Summary of the results of recurrent case*

Case No.	T stage	The number of positive nodes	Expression of p53	Expression of c-erb-B2	PCNA index (%)	FINOR score
1	T ₂	2	—	++	61.2	3.87
2	T ₂	61	—	±	56.8	2.84
3	T ₂	29	+	++	51.8	3.84
4	T ₂	5	—	—	33.7	1.38
5	T ₁	0	—	—	59.0	3.63
6	T ₃	1	—	—	35.8	3.23
7	T ₂	18	—	—	50.8	3.62
8	T ₃	9	—	—	44.8	3.92
9	T ₂	1	—	—	46.8	1.64
10	T ₃	0	—	+	59.3	2.13
11	T ₂	25	—	—	46.7	2.92
12	T ₂	15	++	±	75.8	4.26
13	T ₂	10	++	++	43.3	3.61
14	T ₁	0	+	±	69.6	3.45
15	T ₂	10	—	—	45.7	2.25
16	T ₁	0	—	—	42.6	2.18
17	T ₂	13	+	+	70.6	3.09
18	T ₂	0	—	±	28.5	3.21
19	T ₂	0	—	—	26.4	2.92
20	T ₃	7	—	±	37.7	2.21
21	T ₂	4	++	++	77.8	2.64

Table 4. *Summary of the results of non-recurrent case*

Case No.	T stage	The number of positive nodes	Expression of p53	Expression of c-erb-B2	PCNA index (%)	FINOR score
22	T ₁	0	—	±	70.0	3.04
23	T ₁	0	—	±	25.0	1.42
24	T ₁	0	—	±	24.0	2.26
25	T ₂	1	—	—	38.0	2.11
26	T ₂	0	—	—	71.4	1.97
27	T ₁	0	—	+	25.2	1.89
28	T ₂	0	—	—	13.0	1.96
29	T ₂	1	—	++	13.0	2.25
30	T ₁	0	—	—	25.2	2.75
31	T ₁	0	—	—	52.2	3.30
32	T ₁	6	±	++	30.4	1.44
33	T ₁	0	—	—	16.5	2.52
34	T ₂	1	+	+	44.7	2.83
35	T ₂	0	—	—	21.5	2.16
36	T ₁	0	—	—	29.6	2.08
37	T ₂	13	—	—	36.0	2.28
38	T ₁	0	—	—	52.1	2.34
39	T ₁	0	+	—	85.2	3.80
40	T ₁	0	—	—	47.3	2.18
41	T ₁	0	—	—	23.4	1.81
42	T ₂	0	—	±	42.3	2.74
43	T ₁	2	—	—	26.2	2.98
44	T ₂	0	—	—	41.1	2.29
45	T ₂	0	—	—	45.0	2.19
46	T ₁	0	++	±	31.1	2.41
47	T ₂	1	—	—	46.2	2.11

histochemically detectable p53 protein. Positive ratios of p53 in recurrent and non-recurrent cases were 28.6% (6/21) and 15.4% (4/26), respectively. The staining score of p53 of each case is shown in Tables 3 and 4. The average scores of staining scores of p53 in both groups were 0.71 ± 1.19 and 0.31 ± 0.79 , respectively (Table 5). Both values of recurrent cases had a tendency to be high than those of non-recurrent cases, but the difference was not statistically significant by chi-square test. All normal breast tissues were negative.

In c-erbB-2 staining, twenty out of forty-seven cases (42.6%) showed variable positive immunoreactivity in tumor cell membrane (Fig. 2-A, B), and the other twenty-seven cases (57.4%) were completely negative. Cyto-

plasmic staining was occasionally seen. Positive ratios of c-erbB-2 in recurrent and non-recurrent cases were 52.4% (11/21) and 34.6% (9/26), respectively. The staining score of c-erbB-2 of each case is shown in Tables 3 and 4. The average staining scores of c-erbB-2 in both groups were 1.00 ± 1.18 and 0.58 ± 0.95 , respectively. Both values of recurrent cases were also higher than those of non-recurrent cases. However, no significant differences between the two groups in positive ratio and staining score of c-erbB-2 were detected by chi-square test. All normal breast tissues were negative.

In all cases, immunoreactivity of PCNA was detected in tumor cell nuclei (Fig. 3-A, B). PCNA index of each case is shown in Tables 3 and 4. The average PCNA indices in recurrent and non-recurrent cases were 47.8 ± 14.1 and 39.4 ± 18.2 , respectively (Table 5). There were no significant differences between the two groups in PCNA index by t test. The average PCNA index in normal breast epithelium was 8.36 ± 3.05 , and it was significantly lower than those in recurrent and non-recurrent cases.

Table 5. Comparison of the results between recurrent and non-recurrent cases

	Recurrent case	Non-recurrent case
Lymph node score	10.0 ± 14.5	$0.96 \pm 2.75^*$
T stage	2.05 ± 0.59	$1.42 \pm 0.50^{**}$
p53 score	0.62 ± 1.20	0.12 ± 0.49
c-erbB-2 score	0.92 ± 1.19	0.59 ± 0.87
PCNA index	47.8 ± 14.2	39.5 ± 18.2
FINOR score	3.03 ± 0.78	$2.32 \pm 0.54^*$

Mean $\pm$ S.D.

*Significantly different by t test ($p < 0.01$)

**Significantly different by chi square test ($p < 0.05$)

Observation of FINORs by confocal laser scanning microscope (CLSM)

The green fluorescence of morin chelated with aluminum bound to NORs-associated proteins was seen within the tumor cell nuclei by blue excitation light. It was stable without any detectable bleaching during long-standing observation under the confocal laser scanning microscope (CLSM). We observed FINORs images of

Fig. 1. Immunohistochemical staining with anti-p53 monoclonal antibody. **A:** recurrent case (case 12), **B:** non-recurrent case (case 46). Both cases contain many positive cell nuclei (**A:** ++, **B:** ++). But the staining intensity of recurrent case is stronger than that of non-recurrent case. (Magnification $\times 580$.)

Fig. 2. Immunohistochemical staining with anti-c-erbB-2 monoclonal antibody. **A:** recurrent case (case 3), **B:** non-recurrent case (case 29). In both cases, neoplastic cell membranes are diffusely positive (**A:** ++, **B:** ++). Weak cytoplasmic staining is observed in some tumor cells in both cases. Staining intensity of recurrent case is also stronger than that of non-recurrent case. (Magnification $\times 580$).

Fig. 3. Immunohistochemical staining with anti-PCNA monoclonal antibody. **A:** recurrent case (case 3), **B:** non-recurrent case (case 22). In both cases, many tumor cell nuclei are positively stained. (Magnification $\times 580$).

Fig. 4. FINORs images of nine consecutive sections recorded at $1\ \mu\text{m}$ intervals in the Z direction. **A:** recurrent case (case 12), **B:** non-recurrent case (case 29).

Fig. 5. Three-dimensional reconstruction images of Figure 4 displayed by stereo pair. **A:** recurrent case (case 12), **B:** non-recurrent case (case 29).

nine consecutive sections recorded at $1\ \mu\text{m}$ intervals in the Z direction (Fig. 4-A, B). Three-dimensional reconstruction of these images were displayed by two colors or stereo pair (Fig. 5-A, B). FINORs score of each case is shown in Tables 3 and 4. The average FINORs scores of recurrent and non-recurrent cases were 3.03 ± 0.78 and 2.32 ± 0.54 , respectively (Table 5). There were significant differences between the two groups in FINORs score by t test ($p < 0.01$). Moreover, markedly large NORs with bizarre configuration were often observed in tumor cell nuclei of recurrent cases (Figs. 4-A, 5-A). The average FINORs score in normal breast epithelium was 1.43 ± 0.22 , and it was significantly lower than those in recurrent and non-recurrent cases.

IV. Discussion

There are many reports about prognostic factors and/or proliferative activities of human breast carcinoma, but comparative studies of them between recurrent and non-recurrent breast carcinoma are few. In the present study we compared the axillary lymph node status, T stage, expression of p53 and c-erbB-2, PCNA index, and FINORs score between twenty-one recurrent and twenty-six non-recurrent cases of human breast carcinoma. As a result, there were significant differences between the two groups in lymph node status, T stage, and FINORs score.

The prognosis of patients presenting with operable breast carcinoma is extremely variable and lymph node stage, tumor size, histological grade and tumor type are currently recognized as the most powerful predictors of outcome [27]. The risk of recurrence is increased with the number of lymph nodes involved [18]. The same observation was made in this study.

The percentage of p53 immunoreactive case in the present study was 21.3% (10/47). It is somewhat lower than that of reported in the literature (ranging from 23% to 48%) [5, 15, 20, 28]. This difference may reflect the heterogeneity of the patient populations investigated. The variable prevalence of p53 immunoreactivity may also depend on the choice of different antibodies used for immunocytochemical studies. Positive ratios, staining scores, and staining intensity of p53 in recurrent cases had a tendency to be high and strong than those in non-recurrent cases, although no statistical difference is detected between the two groups. These results are comparable to those reported by Bosari *et al.* [5] and Caleffi *et al.* [6].

c-erbB-2 protein immunohistochemical overexpression was seen in 42.6% cases (20/47). This value correlates with the percentage of positivity reported by Wright *et al.* [34] (45%). There is some diversity of opinion among investigators concerning prognostic significance of c-erbB-2 overexpression in breast carcinoma. In the present study, positive ratios, staining scores, and staining intensity of c-erbB-2 in recurrent cases were higher and stronger than those in non-recurrent cases, but there were no significant differences between the two groups.

The determination of cellular proliferation can be effectively used to predict the relapse and/or death of patients with breast carcinoma. In fact, uncontrolled cell proliferation is one of the most evident factors related to malignancy [17]. We used PCNA index and FINORs score instead of AgNORs score for markers of cell proliferation.

Some studies have found that PCNA is related to poor prognosis [13, 22, 25, 33]. But some authors have found that it does not correlate with prognosis [10, 11]. Our data showed no significant differences in PCNA indices between recurrent and non-recurrent cases. They seem to support the latter results.

We developed a new method of fluorescent staining of nucleolar organizer regions (FINORs) [14]. This method enabled us to observe three-dimensional structures of NORs by using thick sections under confocal laser scanning microscope (CLSM). We found a remarkable increase of FINORs not only in number, but also in volume in the process of tumor progression [14]. Toikkanen *et al.* [26] reported that AgNOR counts had no prognostic value in breast cancer. But in the present study, we demonstrated FINORs scores in recurrent cases were significantly higher than those in non-recurrent cases. Moreover, large FINORs with bizarre configuration were often detected in recurrent cases. These findings indicate that FINORs-CLSM technique may be useful as prognostic factor in patient with breast carcinoma. We could not carry out quantitative analyses of volume and shape irregularity of FINORs. If the softwares for three-dimensional morphometry are more improved, it would be able to analyze these parameters quantitatively. The reasons for the occurrence of large and bizarre NORs in malignancy together with a numerical increase are not fully understood at the present time, but the accumulation of NORs-associated proteins in both intra- and extra-nucleolar spaces might indicate an abnormal nuclear storage of these proteins.

In conclusion, our results indicate that lymph node status, tumor stage, and FINORs score may be useful as risk factors of recurrence of human breast carcinoma. To clarify their prognostic significance in patient with breast carcinoma, further analyses using a larger series of cases seem to be required.

V. References

- Allred, D. C., Clark, G. M., Molina, R., Tandon, A. K., Schnitt, S. J., Gilchrist, K. W., Osborne, C. K., Tormey, D. C. and McGuire, W. L.: Overexpression of Her-2/neu and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. *Hum. Pathol.* 23; 974-979, 1992.
- Bartek, J., Bartkova, J., Vojtesk, B., Staskova, Z., Lukas, J., Rejthar, A., Kovarik, J., Midgley, C. A., Gannon, J. V. and Lane, D. P.: Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. *Oncogene* 6; 1699-1703, 1991.
- Battersby, S. and Andersson, T. J.: Correlation of proliferative activity in breast tissue using PCNA/cyclin. *Hum. Pathol.* 21; 781, 1990.
- Berns, E. M., Klijn, J. G. M., van Staveren, I. L., Portengen, H., Noordgraaf, E. and Foekens, J. A.: Prevalence of amplification of the oncogenes c-myc, HER2/neu, and int-2 in one thousand human breast tumors: correlation with steroid receptors. *Eur. J. Cancer* 28; 697-700, 1992.
- Bosari, S., Lee, A. K. C., Viale, G., Heatley, G. J. and Coggi, G.: Abnormal p53 immunoreactivity and prognosis in node-negative breast carcinomas with long-term follow-up. *Virchows Arch. [A]* 421; 291-295, 1992.
- Caleffi, M., Teague, M. W., Jensen, R. A., Vnencak-Jones, C. L., Dupont, W. D. and Parl, F. F.: p53 gene mutations and steroid receptor status in breast cancer. *Cancer* 73; 2147-2156, 1994.
- Dawson, A. E., Norton, J. A. and Weinberg, D. S.: Comparative assessment of proliferation and DNA content in breast carcinoma by image analysis and flow cytometry. *Am. J. Pathol.* 136; 1115-1124, 1990.
- Egan, M. J. and Crocker, J.: Nucleolar organizer regions in pathology. *Br. J. Cancer* 65; 1-7, 1992.
- Friedrichs, K., Gluba, S., Eidtmann, H. and Jonat, W.: Overexpression of p53 and prognosis in breast cancer. *Cancer* 72; 3641-3647, 1993.
- Gasparini, G., Boracchi, P., Verderio, P. and Bevilacqua, P.: Cell kinetics in human breast cancer: Comparison between the prognostic value of the cytofluorimetric S-phase fraction and that of the antibodies to Ki-67 and PCNA antigens detected by immunocytochemistry. *Int. J. Cancer* 57; 822-829, 1994.
- Gasparini, G., Meli, S., Pozza, F., Cazzavillan, S. and Bevilacqua, P.: PC-10 antibody to proliferating-cell nuclear antigen (PCNA) is not related to prognosis in human breast carcinoma. *Growth Reg.* 12; 145-150, 1992.
- Giri, D. D., Nottingham, J. F., Lawry, J., Dundas, S. A. C. and Underwood, J. C. E.: Silver-binding nucleolar organizer regions (AgNORs) in benign and malignant breast lesions: correlations with ploidy and growth phase by DNA flow cytometry. *J. Pathol.* 157; 307-313, 1989.
- Haerslev, T. and Jacobsen, G. K.: Proliferating cell nuclear antigen in breast carcinomas. An immunohistochemical study with correlation to histopathological features and prognostic factors. *Virchows Arch. [A]* 424; 39-46, 1994.
- Imamura, Y., Noriki, S., Tsuzuki, H., Nitta, Y. and Fukuda, M.: Fluorescent staining of nucleolar organizer regions for three-dimensional display by confocal laser scanning microscope. *Eur. J. Histochem.* 37; 321-328, 1993.
- Iwaya, K., Tsuda, H., Hiraide, H., Tamaki, K., Tamakura, S., Fukutomi, T., Mukai, K. and Hirohashi, S.: Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. *Jpn. J. Cancer Res.* 82; 835-840, 1991.
- Malinin, G. I.: Permanent fluorescent staining of nucleic acids in isolated cells. *J. Histochem. Cytochem.* 26; 1018-1020, 1978.
- Miller, W. R.: Prognostic factors in breast cancer. *Br. J. Cancer* 66; 775-776, 1992.
- Nemoto, T., Vana, J., Bedwani, R., Baker, H. W., McGregor, F. H. and Murphy, G. P.: Management and survival in female breast cancer. *Cancer* 15; 2917-2924, 1980.
- Saccani Jotti, G.: New prognostic indicators in resectable breast cancer. *Anticancer Res.* 49; 1227-1232, 1989.
- Shrestha, P., Yamada, K., Wada, T., Maeda, S., Watatani, M., Yasutomi, M., Takagi, H. and Mori, M.: Proliferative cell nuclear antigen in breast lesions: correlation of c-erbB-2 oncoprotein and EGF receptor and its clinicopathological significance in breast cancer. *Virchows Arch. [A]* 421; 193-202, 1992.

21. Siitonen, S. M., Isola, J. J., Rantala, I. S. and Helin, H. J.: Intratumor variation in cell proliferation in breast carcinoma as determined by anti-PCNA monoclonal antibody and automated image analysis. *Am J. Clin. Pathol.* 99; 226–231, 1993.
22. Siitonen, S. M., Kallioniemi, O. P. and Isola, J. J.: Proliferating-cell-nuclear-antigen immunohistochemistry using monoclonal antibody 19A2 and a new antigen-retrieval technique has prognostic impact in archival paraffin-embedded node-negative breast cancer. *Am. J. Pathol.* 142; 1081–1089, 1993.
23. Smith, R. and Crocker, J.: Evaluation of nucleolar organizer-associated proteins in breast malignancy. *Histopathology* 12; 113–125, 1988.
24. Suzuki, K., Katoh, R. and Kawaoi, A.: Immunohistochemical demonstration of proliferating cell nuclear antigen (PCNA) in formalin-fixed, paraffin-embedded sections from rat and human tissues. *Acta Histochem. Cytochem.* 25; 13–21, 1992.
25. Tahan, S. R., Neuberger, D. S., Dieffenbach, A. and Yacoub, L.: Prediction of early relapse and shortened survival in patients with breast cancer by proliferating-cell-nuclear-antigen score. *Cancer* 71; 3552–3559, 1993.
26. Toikkanen, S. and Joensuu, H.: AgNOR counts have no prognostic value in breast cancer. *J. Pathol.* 169; 251–254, 1993.
27. Todd, J. H., Dowle, C., Williams, M. R., Elston, C. W., Ellis, I. O., Hinton, C. P., Blamey, R. W. and Haybittle, J. L.: Confirmation of a prognostic index in primary breast cancer. *Br. J. Cancer* 56; 489–492, 1987.
28. Tsuda, H., Hirohashi, S., Shimosato, Y., Hirota, T., Tsugane, S., Yamamoto, H., Miyajima, N., Toyoshima, K., Yamamoto, T., Yokota, J., Yoshida, T., Sakamoto, H., Terada, M. and Sugimura, T.: Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: hst-1/int-2 and c-erbB-2/ear-1. *Cancer Res.* 49; 3104–3108, 1989.
29. Tubiana, M. and Courdi, A.: Cell proliferation kinetics in human solid tumors: Relation to probability of metastatic dissemination and long-term survival. *Radiother. Oncol.* 15; 1–18, 1989.
30. Tubiana, M., Pejovic, M. H., Kosciely, S., Chavaudra N. and Malaise, E.: Growth rate, kinetics of tumor cell proliferation and long-term outcome in human breast cancer. *Int. J. Cancer* 44; 17–22, 1989.
31. Vogelstein, B. and Kinzler, K. W.: p53 function and dysfunction. *Cell* 70; 523–526, 1992.
32. Waseem, N. H. and Lane, D. P.: Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. *J. Cell Sci.* 96; 121–129, 1990.
33. Wolman, S. R., Pauley, R. J., Mohamed, A. N., Dawson, P. J., Visscher, D. W. and Sarkar, F. H.: Genetic markers as prognostic indicators in breast cancer. *Cancer* 1992; 70: 1765–1774.
34. Wright, C., Angus, B., Nicholson, S., Sainsbury, J. R. C., Cairns, J., Gullick, W. J., Kelley, P., Harris, A. L. and Horne, C. H. W.: Expression of c-erbB-2 oncoprotein: a prognostic indicator in human breast cancer. *Cancer Res.* 49: 2087–2090, 1989.
35. Zhou, D. J., Ahuja, H. and Cline, M. J.: Proto-oncogene abnormalities in human breast cancer: c-erbB-2 amplification does not correlate with recurrence of disease. *Oncogene* 4; 105–108, 1989.