Acta Med. Nagasaki 38:226 – 231

Studies on the Utility of Sequential Staining Technique using PCNA and AgNORs for Assessing the Degree of Malignancy of Gastric Carcinoma

Toshikazu Matsuo

The First Department of Surgery, Nagasaki University School of Medicine Nagasaki, Japan.

In thirty cases of resected primary gastric carcinoma, the relative utility of argyrophilic nucleolar organizer regions (AgNORs) staining (single staining) and sequential staining technique using proliferating cell nuclear antigen (PCNA) and AgNORs was studied.

The mean number of AgNORs in PCNA-positive cells was significantly larger than that in AgNORs single stained cells. For above two groups, correrating mean number of AgNORs with clinicopathological factors revealed a significant difference between stage I + II and stage III + IV. In lymph node metastasis, the depth of invasion and size, only sequential stain-positive cells showed significant difference.

On the other hand, only that of sequential stain-negative cells, a significant difference was found with regard to liver metastasis.

As for other assessment, the ratio of AgNORs area per nuclear area was investigated using an image analyzer (IBAS 2000 KONTRON). Comparison of the ratio of AgNORs area per nuclear area between PCNA-positive cells and AgNORs single stained cells revealed a significant difference in regard to lymph node metastasis. On the other hand, between the ratio of AgNORs area per nuclear area in PCNA-negative cells and that of AgNORs single stained cells, a significant difference was found in regard to liver metastasis.

Thus, the evaluation using PCNA for classifying cells into proliferating cells and nonproliferating cells in combination with AgNORs staining provided information of greater values to the malignancy assessment of gastric carcinoma as compared with the standard AgNORs single staining.

Introduction

The malignancy assessment of carcinoma cells based on their proliferating potential using growth-related markers such as PCNA, Ki-67 and DNA polymerase α has been much studied in various organs by employing immunohistochemical techniques.

Nucleolar organizer regions (NORs) are loops DNA in which ribosomal RNA (rRNA) is encoded. NOR-associated proteins and NORs themselves have been visualised recently using the one-step silver staining (AgNORs) method. The silver staining technique identifies non-histone nuclear proteins associated with the sites of rRNA transcription, with an increase in AgNORs number and area are said to reflect the proliferative activity of cells. Compared with normal cells, carcinoma cells have a higher proliferation potential, and the higher the proportion of cells in proliferating phase, the poorer is the prognosis of the cases. It is, therefore, considerd that their staining methods are of great value for assessing the degree of malignancy in gastric carcinoma.

However, in order to evaluate all the biological behaviors of carcinoma cells, not only cells in proliferating phase but also cells in non-proliferating phase (resting cells) must be taken into consideration. This is because, in actual chemotherapy, non-proliferating cells are much refractory to treatment, and information on nonproliferating cells may be of great clinical value. From this point of view, the author stained proliferating cells with PCNA and, at the same time, AgNORs in both the proliferating population and the non-proliferating population, and evaluated the cytological characteristics of the cells in the respective population.

Materials and Methods

Materials

Samples of primary gastric carcinoma were available from 30 patients who underwent gastrectomy at the First Department of Surgery of Nagasaki University School of Medicine. No patient had received chemotherapy before surgery.

Methods

A cube, 1cm^3, was cut out from the carcinoma lesion, fixed in 10% neutral buffered formalin for not more than 24 hours and embedded in paraffin. Sections were cut at 3 μm thickness for use as specimens to be stained. The rationale of the above procedure was as follows. PCNA is variable in staining intensity and rate according to the fixation procedure and time. When a paraffin block from a usual surgical specimen fixed for a prolonged time in 10% formalin is employed, immunostaining with PCNA may not necessarily yield stable results but tends to give negative reaction.[4] The author also tentatively stained usual surgical

specimens fixed in 10% formalin in the conventional manner as well as specimens cut into 1cm^3 cubes and fixed in 10% neutral buffered formalin for less than 24 hours and discovered a remarkable difference between the two kinds of specimens in staining intensity and rete. Therefore, in the present study, the latter fixation procedure was followed with satisfactory results. In sequential staining technique, PCNA was stained first, followed by AgNORs staining.

1. Immunohistochemical staining with PCNA.
As the primary antibody, PC10 (DAKO), and anti-PCNA mouse monoclonal antibody, was used in 100-fold dilution for 60 minutes at room temperature. The staining was performed by the labelled streptavidine-biotin (LSAB) method using DAKO LSAB KIT, Alkaline Phosphatase. As the chromogen, Fast Red was used.

2. AgNORs staining
The PCNA stained preparation was washed thoroughly with distilled water (DW3) and AgNORs staining was carried out by the one-step silver staining method. The staining solution was prepared by dissolving powdered gelatin at a concentration of 2% (W/V) in de-ionized water, pure fornic acid was then added to a final concentration of 1%. This solution was mixed 1:2 volumes with 50% aqueous silver nitrate solution to give the working solution. The sections were incubated with the solution in the darkness for 50 minutes. The AgNORs single staining was also carried out in the same menner except that reaction time was 25 minutes. To facilitate the determination of the number and sectional area of AgNORs, the silver was decolorized with 5% sodium thiosulfate in both procedures.

Methods of assessment

In each case, using a light microscope (Olympus VANOX) at a magnification of x1000, 100 carcinoma cells were observed and the mean numbers of AgNORs, granules per nucleus for each of AgNORs single stained carcinoma cells and PCNA-positive and PCNA-negative cells were calculated.

Furthermore, in each case, using an image analyzer (IBAS2000, Carl Zeiss), 100 carcinoma cells were scanned and the mean nuclear sectional area, AgNORs count, mean sectional area of AgNORs and the ratio of AgNORs per nuclear area were calculated for each of AgNORs single stained carcinoma cells and PCNA-positive and PCNA-negative cells. A brief description of the image analyzer may be pertinent on this occasion. Once the area to be examined by microscopy is selected under a x1000 oilimmersion lens, the image is photographed with color CCD cameras connected to the barrel of the microscope and displayed on the cathode-ray tube and this live picture image is input to the image analyzer. Thereafter, the nuclei are traced. For recognition of the whole nucleus, binarization is performed at a comparatively low density. Then, for identification of NORs, binarization is carried out at high density. The above procedure is repeated for 100 nuclei per specimen and the data are printed out. The data are tabulated as histograms. The statistical analysis of correlates of the data with clinicopathological factors and DNA ploidy was performed by the Wilcoxon test.

Results

The breakdown of 30 gastric carcinoma cases is presented in Table 1. The criteria used were in conformity with the Gastric Cancer Staging System. In sequential staining technique, the PCNA labelling index (LI) was 46.3% and there was no correlation with clinicopathological factors and DNA ploidy. PCNA-positive cells included both strongly positive and weakly positive cells. In PCNA staining, the nuclei were stained red and the intensity of stain was varying much. AgNORs were observed as small black dots and large irregularly-shaped black dots in the nucleus. A typical sequential staining technique case is shown in Fig. 1.

Table 1. Pathologic profiles and DNA ploidy of the patients with gastric carcinoma

No. of cases		30
Macro	0	4
	1	2
	2	7
	3	10
	4	5
	5	2
Histologic type	tub 1	5
	tub 2	10
	por	15
Depth	ps (–)	6
	ps (+)	24
stage	I	5
	II	1
	III	12
	IV	12
DNA ploidy	Diploid	10
	Aneuploid	20

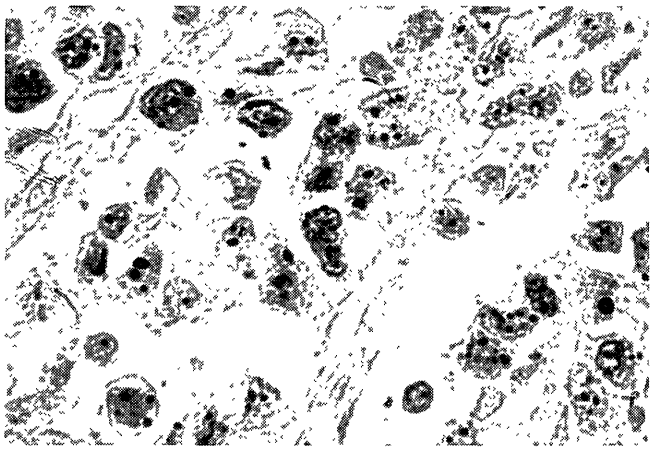

Fig. 1. The sequential stain technique using PCNA and AgNORs.

1. Evaluation of AgNORs counts

The mean AgNORs counts of PCNA-positive cells, PCNA-negative cells and AgNORs single stained cells were 8.28 ± 1.45, 3.62 ± 0.67 and 5.76 ± 1.04, respectively. Thus, a significant difference ($P < 0.0001$) was found between PCNA-positive and -negative cells and between PCNA-positive cells and AgNORs single stained cells (Table 2).

Table 2. Results (mean AgNOR counts)

PCNA positive cells	8.28 ± 1.45	※ ※
PCNA negative cells	3.62 ± 0.67	
AgNORs single stained cells	5.76 ± 1.04	

※ $P < 0.0001$

The correlates with clinicopathological factors (according to the Gastric Cancer Staging System) and DNA ploidy were studied. As to size, the mean value of 30 caces, viz. 7.2cm, was used as the test. Compared with AgNORs single staining, the mean AgNORs counts of PCNA-positive cells showed a statistically significant difference between stage I + II and stage III + IV at a lower level of significance. In regard to lymph node metastasis, depth of invasion and size, only the mean AgNORs counts of PCNA-positive cells showed a significant difference. The mean AgNORs counts of PCNA-negative cells showed a significant difference in involvement of liver metastasis. With regard to DNA ploidy, aneuploid showed a tendency of increase as compared with diploid but there was no significant difference in any population (Table 3).

2. Quantitative evaluation of AgNORs area.

The mean AgNORs sectional area (μm^2) of normal cells, PCNA-positive and -negative carcinoma cells and AsNORs single stained carcinoma cells were 1.56 ± 0.48, 10.14 ± 2.35, 4.08 ± 0.99 and 7.59 ± 2.34, respectively. There was significant differences among all populations ($P < 0.0001$) (Table 4).

Table 4. Mean AgNOR area for each cells groups (μm^2)

PCNA positive cells	10.14 ± 2.35	※ ※
PCNA negative cella	4.08 ± 0.99	
AgNORs single stained cells	7.59 ± 2.34	

(normal cells 1.56 ± 0.48) ※ $P < 0.0001$

In the sectional area of nuclei, too, normal cell and PCNA- positive and -negative cells and AgNORs single stained carcinoma cells were 28.13 ± 2.23, 78.15 ± 17.76, 60.42 ± 13.2 and 67.56 ± 18.75, respectively. There were significant differnces among all population. (Table 5) Therefore, the sectional area of AgNORs relative to the sectional area of nuclei was investigated to find the ration of AgNORs area per nuclear area (%).

The ratio of AgNORs area per nuclear area (%) of

Table 3. Relationship between clinicopathologic factors and mean AgNOR counts

	A		B		C	
PS–	6.79 ± 1.72	※※	5.09 ± 1.16	NS	3.41 ± 0.81	NS
PS+	8.65 ± 1.12		5.92 ± 0.95		3.67 ± 0.63	
stage I II	6.53 ± 1.30	※	4.86 ± 0.80	※※	3.44 ± 0.84	NS
stage III IV	8.72 ± 1.12		5.98 ± 0.97		3.66 ± 0.62	
size ≦ 7.2cm	7.45 ± 1.46	※	5.33 ± 1.02	NS	3.56 ± 0.64	NS
size > 7.2cm	9.00 ± 0.99		6.12 ± 0.93		3.67 ± 0.70	
n (–)	7.23 ± 1.57	※※	5.14 ± 0.96	NS	3.49 ± 0.68	NS
n (+)	8.73 ± 1.15		6.01 ± 0.97		3.67 ± 0.66	
H –	8.20 ± 1.42	NS	5.61 ± 0.86	NS	3.45 ± 0.52	※※
H +	8.65 ± 1.67		6.48 ± 1.58		4.42 ± 0.77	
Diploid	7.94 ± 1.10	NS	5.38 ± 0.91	NS	3.45 ± 0.64	NS
Aneuploid	8.45 ± 1.59		5.94 ± 1.06		3.70 ± 0.67	

A: PCNA positive cells
B: AgNORs single stained cells
C: PCNA negative cells

※ P < 0 01
※※ P < 0.05
NS: significant

Table 5. Mean nuclear area for each cells groups (μm^2)

PCNA positive cells	78.15 ± 17.76	※
PCNA negative cells	60.42 ± 13.20	※※
AgNORs single stained cells	67.56 ± 18.75	

(normal cells 28.13 ± 2.23) ※ $P < 0.0001$ ※※ $P < 0.001$

normal cells, PCNA-positive and -negative carcinoma cells and AgNORs single stained carcinoma cells were 5.73 ± 1.54, 13.13 ± 2.20, 6.85 ± 1.47, 11.38 ± 2.26, respectively, with a significant difference ($P < 0.0001$) among them (Table 6).

Table 6. The ratio of AgNORs area per nuclear area for each cells groups (%)

PCNA positive cells	13.13 ± 2.20	※
PCNA negative cells	6.85 ± 1.47	※
AgNORs single stained cells	11.38 ± 2.26	

(normal cells 5.73 ± 1.54) ※ $P < 0.0001$

There results were evaluated in correlation with clinicopathological factors and DNA ploidy. As a result, only in lymph node metastasis, the ratio of AgNORs area per nuclear area (%) of PCNA-positive cells showed a significant differnce ($P < 0.05$) from that of AgNORs single stained cells.

In the assessment of PCNA-negative cells, a significant differnence ($P < 0.01$) was found in cases of liver metastasis. There was no correlation with DNA ploidy in any population (Table 7).

Discussion

The sequential staining technique of gastric carcinoma cells was carried out using AgNORs and PCNA. The AgNORs staining was originally developed by Howell *et al* in 1980[1] and subsequently sophisticated by Ploton *et al* in 1986.[2] Since then, with Crocker *et al* as leaders, the validity of the technique has been extensively explored, mainly in malignant tumors, in various professional fields. In fact, because the resulting count, size and morphological information reflect the proliferation, differentiation and metabolism of cells, this staining technique has been utilized as a measure of malignancy.

PCNA, a growth-related marker, can be used for paraffin sections and have been employed broadly in retrospective studied, too. It is known that PCNA is a non-histon protein with a molecular weight of 36KD and that it is synthesised in the cell nucleus in the late G1 and S-phase of the cell cycle and functions as auxiliary protein of DNA polymerase δ.[3]

While this PCNA staining technique is theoretically designed for the identification of proliferating cells, weakly positive cells were also regarded as positive cells in the present investigation. The rationale is that since the PCNA protein is relatively stable, it probably stays for many hours in the nucleus and that, though at low levels, it is present in G2 and M phase as well. Therefore, in the present study, PCNA-positive cells were regarded as cells of proliferating phase,[5,6] and in the sequential staining technique, PCNA was used only as a means for classifying cells into proliferating and non-proliferating phase.

As the established theory employing AgNORs single staining for malignancy assessment, we know of the report of Crocker et al in 1987 which describes that in non-Hodgkin's lymphoma the AgNORs count showed a significant difference between low-grade lymphoma and high-grade lymphoma.[7] However, it has also been reported that in solid tumors such as colorectal carcinoma and breast carcinoma, AgNORs single staining alone does not provide differentiation with statistical significance in pathological factors and cannot be a self-contained prognostic factor.[8,9]

Table 7. Relationship between clinicopathologic factors and the ratio of AgNORs area per unclear area

	A		B		C	
PS–	11.46 ± 1.61	※	9.50 ± 2.01	※	6.16 ± 1.50	NS
PS+	13.55 ± 2.15		11.86 ± 2.09		7.02 ± 1.44	
stage I II	11.60 ± 1.86	NS	9.82 ± 2.40	NS	6.27 ± 1.57	NS
stage III IV	13.51 ± 2.14		11.78 ± 2.09		6.99 ± 1.44	
size ≦ 7.2cm	12.48 ± 1.83	NS	11.35 ± 2.31	NS	6.76 ± 1.07	NS
size > 7.2cm	13.70 ± 2.38		11.42 ± 2.29		6.93 ± 1.78	
n (–)	11.72 ± 1.69	※※	10.56 ± 2.41	NS	6.16 ± 1.30	NS
n (+)	13.73 ± 2.14		11.74 ± 2.15		7.15 ± 1.46	
H –	13.02 ± 2.12	NS	11.33 ± 2.25	NS	6.45 ± 1.01	※
H +	13.67 ± 2.78		11.64 ± 2.52		8.85 ± 1.89	
Diploid	12.83 ± 2.70	NS	10.91 ± 2.61	NS	6.68 ± 0.88	NS
Aneuploid	13.20 ± 1.96		11.62 ± 2.09		6.93 ± 1.70	

A: PCNA positive cells ※ $P < 0.01$
B: AgNORs single stained cells ※※ $P < 0.05$
C: PCNA negative cells

Therefore, efforts are being made to develop an accurate yardstick of malignancy by using a variety of growth-related markers in combination. For example, Kakeji *et al* reported that a combination of AgNORs and Ki-67 in gastric carcinoma provided a prediction of lymph node metastasis.[10] However, this is not an assessment made on the same cell.

As to the simultaneous assessment in the same section by AgNOR-Ki-67 sequential staining, Murray suggested its feasibility in 1989.[11] Then Janmohamed *et al* practiced the technique in non-Hodgkin's lymphomas in 1990 and reported that the mean AgNORs count of Ki-67-positive cells was significantly higher than that of Ki-67-nepative cells.[12] In 1993 F. G. Smith et al reported about correlation between PCNA and AgNOR scores in non-Hodgkin's lymphomas using sequential staining technique.[13] In this paper, they reported, altough we have shown an association between PCNA labelling and AgNOR counts in NHL and therefore, yet again, an association between cell proliferation and AgNOR scores, it appears that PCNA negative cells still have relatively high counts in lymphomas. As for the solid tumor, in 1993, Yoshida *et al* established a PCNA-AgNORs sequential staining technique using colorectal carcinoma cells.[14] However, their paper is primarily devoted to fundamental studies on the staining method and considers little about correlation of data with clinicopathological factors. Under the circumstances, the present author used this technique in gastric carcinoma and evaluated the data in correlation with clinicopathological factors for the first time. As a result, the mean AgNORs count of the sequential stain-positive cells showed a statistical difference at a lower P value of significance between stage I + II and stage III + IV. Moreover, only sequential stain-positive cells showed significant differences in lymph node mtastasis, depth of invasion and size.

In PCNA- negative cells, a significant difference was found in the incidence of liver metastasis. These findings could not be generated by the standard AgNORs single staining technique and the information provided by this sequential staining technique seems to have the potential of being a self-contained prognostic factor.

To resolve the indefiniteness of data interpretation, an image analyzer which permits an objective size assessment of AgNORs was additionally employed in the present study.

Regarding the image analyzer, Derenzini *et al* report that in epithelial tumor of the colon, the carcinoma cells are greater in mean AgNORs count per nucleus and smaller in size than adenoma and hyperplastic polyp.[15] However, there is no report on an investigation using gastric carcinoma cells. In the present study, not only the number but also the sectional area of AgNORs in carcinoma cells showed the significant increase compared with normal cells. One finding of unusual interest was the ratio of AgNORs area per nuclea area in nonproliferating cells showed a significant difference ($P < 0.01$) in liver metastasis cases. While this is true with the mean AgNORs count, it is suspected that a liver metastasis case has a higher proliferation potential even in the cells of nonproliferating phase. This is a new sort of information which cannot be obtained by single staining and may be the admonition that in cases liable to develop liver metastasis attention should be paid to mean AgNORs count and the ratio of AgNORs area pre nuclear area in nonproliferating phase. Should an exploration of this kind be extended to greater cohorts in future studies, we might even be able to establish a critical value.

The question to be addressed to in the future for the sake of this sequential staining technique as applied to gastric carcinoma is concerned with the evaluation of signet ring cell carcinoma. This cell has a significantly low labelling index with PCNA. Although only 3 relevant specimens could be studied in the present investigation, their PCNA labelling index was 10.2% versus 46.3% for the total population. In this connection, it might be the case that as the very nucleus of the cell is fairly small, the accumulation of PCNA is also proportionally small and, therfore, we have to deal with this type of cell separately as a special one.

It is, thus, clear from the above results that in order to evaluate the degree of malignancy of tumors from their proliferation potential, the standard AgNORs single staining method is inadequate and that with attention paid to nonproliferating cells, no to speak of proliferating cells, as many parameters of intracellular information as possible should be unearthed from the same cell by a sequential staining technique such as this. Actually in the present study, useful information was obtained on gastric carcinoma cells in both proliferating and nonproliferating phases and this suggests that the described sequential staining technique is a recommendable procedure.

References

1) Howell WM & Black DA: Controlled silver staining of nucleolus organizer regions with a protective colloidal developer; 1-step method. Experientia, 36:1014-1015, 1980.
2) Ploton. D, Menager, M, Jeannesson. P, Adnet. J. J: Improvement in the staining and in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer regions at the optical level: Histochem J, 18:5-14, 1986.
3) Bravo. R, Frank. R, Blundell. P, et al: Cyclic/PCNA is the auxillary protein of DNA polymerase δ.: Nature, 326:515--517, 1987.
4) Suzuki. K, Katoh. R, Kawaoi. A,: Immunohistchemical demonstration of proliferating cell nuclear antigen (PCNA) in formalin-fixed, paraffin-embedded sections from rat and human tissue. Acta Histochem 25:13-30, 1992.
5) Bravo, R, & Macdonald-Bravo. H: Existense of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites.: J. Cell Biol. 105, 1549-1554, 1987.
6) Kurki. P, Vanderlaan. M, Dolbeare. F, Gray. J & Tan. E. M.: Expression of proliferating cell nuclear antigen (PCNA) /cyclin during the cell cycle. Exp. Cell Res. 166:209-219, 1986.

7) J. Crocker, Paramjet Nar.: Nucleolar organizer regions in lymphomas. Jounal of Pathology. vol. 151:111-118, 1987.
8) Z. Rater, P. Surtees, G. Tildsley and C. Corbishley: The prognostic value of argyrophil nucleolar organizer regions (AgNORs) in colorectal cancer. European Jounal of Surgical Oncology.18:37-40, 1992.
9) N. P. M. Sacks, J. Crocker, R. W. Blamey.: Silver-stained nuclear organizer region counts are of no prognostic value in primary breast cancer. European Jounal of Surgical oncology. 18:98-102, 1992.
10) Yoshihiko Kakeji, Keizou Sugimachi.: Predictive value of Ki-67 and Argyrophilic Nucleolar Organizer Region Staining for lymph node metastasis in Gastric Cancer. Cancer Research. 51:3505-3506, 1991.
11) Murry PG, Boldy DAR, Crocker J & Ayres JG: Sequential demonstration of antigens and AgNORs in frozen and paraffin sections. J. Pathol, 159:169-172, 1989.
12) Janmohamed RMI, Murray PG, Crocker J & Leyland MJ.: Sequential demonstration of nucleolar organizer regions and Ki-67 immunolabelling in non-Hodgkin's lymphomas. Clin. lab. Haemat, 12:395-399, 1990.
13) F. G. Smith, P. G. Murray, J. Crocker: Correlation between PCNA and AgNOR scores in non-Hodgkin's lymphomas using sequential staining technique. J. Clin Pathol, 46:28-31, 1993.
14) Kazuya Yoshida et al.: A study of the sequential demonstration of nucleolar organizer regions and PCNA immunolabelling in colorectal carcinomas.: The Acta Medica Nagasakiensia. vol. 38 No. 1 January 1993.
15) Derentini. M, Romagnoli. T, Mingazzini. P, et al.: Interphatic nucleolar organizer region distribution as a diagnostic parameter to differentiate benign from malignant epithelial tumors of human intestine.: Virchows Archiv. B Cell Pathol. 54:334,1988.