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<td>Author(s)</td>
<td>Yoshida, Kazuya; Tomita, Masao</td>
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A Study of the sequential demonstration of nucleolar organizer regions and PCNA immnolabelling in colorectal carcinomas

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Summary: The possibility of a sequential demonstration of the nucleolar organizer regions (NORs) and proliferating cell nuclear antigen (PCNA) by the argyrophil (AgNOR) method and immunostaining, respectively, was explored in colorectal carcinomas. Both PCNA and the argyrophilic proteins of NORs (AgNORs) were clearly visualized when immunostaining of PCNA by the labelled streptavidine-biotin (LSAB) method was performed before the AgNOR method. A comparative study revealed that the PCNA labelling index determined with sections stained for PCNA alone was 1.8 to 6% greater than that determined by the sequential double staining method. The difference in AgNOR number per nucleus was not statistically significant, suggesting that the double staining method can be used for determining both the AgNOR number and PCNA labelling index.

This double-staining method was used to study AgNORs and PCNA in 12 patients with colorectal cancer. The mean AgNOR numbers for PCNA positive and negative cells were 11.9 and 3.4, respectively. The former number was significantly greater than the later (p < 0.0001), indicating that the interphase AgNOR number determined by the double-staining method reflects the proliferating activity of the cells. Separate enumeration of NORs by this method in dividing and non-dividing cancer cells is expected to provide important information for the analysis of cancer cell kinetics.

Introduction

The nucleolar organizing regions (NORs) are the looped structures of rDNA occurring in chromosomes Nos. 13, 14, 15, 21, and 22. The argyrophilic proteins associated with NORs are referred to as AgNORs. The number, size and distribution have been correlated with the cell’s ability to multiply or divide, and, as a result, biological malignancy of carcinomas. The proliferating cell nuclear antigen (PCNA), also called cyclin, is a non-histone protein measuring 36kD, which accumulates in the nuclei of cells during the late G1 to S phase of the cell cycle. This protein has been known to function as a auxiliary protein of DNA polymerase δ, and has thereby attracted attention as a marker of proliferating cells. In order to demonstrate the association between the AgNOR number and proliferation of cancer cells in colorectal carcinomas, the following basic study on the sequential demonstration of AgNORs and PCNA was performed.

Materials and Methods

Materials

Colorectal carcinomas removed by surgery in this department were studied. The specimens were fixed in 10% formalin and embedded in paraffin. The blocks were cut into slices 3 μm thick and stained.

Immunohistochemical staining of PCNA

Anti-PCNA mouse-monoclonal antibody (PC10:DAKO) was diluted 100 times and used as the primary antibody. To stain PCNA, the DAKO LSAB Kit, Alkaline Phosphatase or DAKO LSAB Kit. Peroxidase was used for the labelled streptavidine-biotin (LSAB) method. The LSAB immunoalkaline phosphatase method was used with New fuchsin, Fast red or Fast blue as the chromogenic substrate. For the LSAB immunoperoxidase method, DAB or AEC was used.

AgNOR staining

The method of Ploton et al. was modified as described below and used to stain AgNORs before and after immunohistochemical staining of PCNA. Briefly, the tissue section was washed in distilled water. The reagent prepared in the dark by combining 1 volume of 2% gelatin solution in 1% formic acid solution and 2 volumes of 50% silver nitrate solution was spread over the section, which was then left at room temperature for varying times between 30 and 60 min to stain AgNORs, and then washed in distilled water.
Results

1) Experiment to establish the optimal conditions for the sequential demonstration

**Conditions of reaction of PCNA with PC10**

The optimal conditions for the reaction of PCNA with PC10 were determined. Four sections cut serially from each of 5 paraffin-embedded blocks were stained for PCNA after immunological reaction with PC10 under the following 4 different conditions: at room temperature for 30 and 60 min and at 4 °C overnight and for 2 days. The LSAB immunoperoxidase method was performed with a chromogenic substrate DAB. More than 1,000 cells were examined for PCNA microscopically using a x40 objective, and the labelling with PC10 index (LI) was determined (Table 1).

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Room temp. 30 min.</th>
<th>Room temp. 60 min.</th>
<th>4°C overnight</th>
<th>4°C 2 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>D690</td>
<td>80.7</td>
<td>90.1</td>
<td>90.2</td>
<td>90.1</td>
</tr>
<tr>
<td>D694</td>
<td>47.3</td>
<td>55.8</td>
<td>89.1</td>
<td>84.2</td>
</tr>
<tr>
<td>D750</td>
<td>60.4</td>
<td>55.9</td>
<td>82.7</td>
<td>74.7</td>
</tr>
<tr>
<td>D791</td>
<td>75.0</td>
<td>82.0</td>
<td>91</td>
<td>90.2</td>
</tr>
<tr>
<td>D879</td>
<td>64.7</td>
<td>82.7</td>
<td>84.7</td>
<td>86.3</td>
</tr>
</tbody>
</table>

The value of LI as determined by the reaction of PCNA with PC10 at 4 °C overnight or for 2 days tended to be greater than that determined at room temperature for 30 or 60 min, and practically no non-specific reaction occurred under any of the reaction conditions. From these results, the immunoreaction of PCNA with PC10 was considered to require incubation at 4 °C overnight or longer.

**Distinction**

When sections were stained for AgNORs as described above, the entire nucleus was dyed slightly brown, resulting in indistinctness of the color was derived from the chromogenic substrate used. In an attempt to make AgNORs noticeable, the sections were processed with a sodium thiosulfate solution; i.e., 5 sections serially cut from each of 5 carcinomas were stained first for PCNA and then AgNORs. The sections were immersed in 5% sodium thiosulfate solution for 5 min, and then washed in distilled water. Three of the 5 sections were stained for PCNA by the LSAB immunoalkaline phosphatase method with New fuchsin, Fast red and Fast blue. The other two were stained by the LSAB immunoperoxidase method with DAB and AEC. All sections were incubated for 60 min for staining AgNORs.

Treatment of the stained sections with a sodium thiosulfate solution effectively removed the brown color in the background, so that the color arising from the chromogenic substrate became distinct.

Of the chromogenic substrates studied, DAB which stained PCNA brown made AgNORs difficult to distinguish. AgNORs and PCNA could be distinguished easily when AEC, New fuchsin, Fast red or Fast blue was used. Fast red producing a clear bright red color was superior to the other chromogenic substrates in facilitating both distinction between PCNA positive and negative cells and recognition of AgNORs in the nuclei of PCNA positive cells.

**Duration of incubation for staining AgNORs**

To test effects of the duration of incubation on visualization in the AgNORs method, serial sections cut from each of 5 carcinomas were stained first for PCNA and then for AgNORs. The sections were then treated with a sodium...
K. Yoshida et al.: A Study of the sequential demonstration thiosulfate solution to make AgNORs distinct. In this experiment, PCNA was stained by the LSAB immunoalkaline phosphatase method with Fast red, and, to stain AgNORs, sections were incubated for 30, 40, 50 or 60 min.

The results showed that, in sections incubated for 30 or 40 min, AgNORs were somewhat difficult to count because of inadequate visualization. When the duration of incubation was prolonged to 50 or 60 min, the color of Ag-NORs become dense and clear, and it was easy to count them. In some of the sections incubated for up to 60 min, however, the nucleolus was stained densely with silver, rendering the AgNORs difficult to distinguish.

These results indicated that the best double staining of PCNA and AgNORs can be performed if sections stained for PCNA by the LSAB immunoalkaline phosphatase method with Fast red are incubated for 50 min to stain AgNORs and then washed in 5% sodium thiosulfate solution for 5 min to make them distinct.

2) Comparison of the AgNOR number determined with sections stained for AgNORs alone with that determined by the double staining method

Two sections were serially cut from each of 5 carcinomas. One of the two sections was incubated for 50 min to stain AgNORs and then treated with 5% sodium thiosulfate solution to make them distinct. The nuclei were then stained with hematoxylin. The other section was stained for AgNORs and PCNA by the double staining method. Both sections were examined with a microscope using a x100 objective. AgNORs were counted among 100 cells, and the number per cell was calculated.

The number determined with sections stained for AgNORs alone was not significantly different from that determined by the double staining method (Table 2).

<table>
<thead>
<tr>
<th>Case no.</th>
<th>PCNA only</th>
<th>PCNA+AgNOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>D761</td>
<td>9.87 ± 4.79</td>
<td>8.76 ± 3.89</td>
</tr>
<tr>
<td>D762</td>
<td>9.48 ± 3.87</td>
<td>9.14 ± 4.33</td>
</tr>
<tr>
<td>D765</td>
<td>9.54 ± 3.19</td>
<td>10.11 ± 4.34</td>
</tr>
<tr>
<td>D773</td>
<td>9.27 ± 3.66</td>
<td>8.62 ± 3.18</td>
</tr>
<tr>
<td>D774</td>
<td>8.59 ± 3.59</td>
<td>9.32 ± 3.30</td>
</tr>
</tbody>
</table>

3) Comparison of the value of LI determined by immunostaining of PCNA alone with that obtained by the double staining method

Two sections were serially cut from each of 5 carcinomas. One of the two sections was stained for PCNA by the LSAB immunoalkaline phosphatase method with Fast red, and then for the nuclei with hematoxylin. The other was stained by the double staining method. These sections were examined under a microscope using a x40 objective. A total of 1,000 or more cells were observed, and the PCNA labelling index was calculated. The value determined with sections stained for PCNA alone was 1.8 to 6% greater than that determined by the double staining method (Table 3).

<table>
<thead>
<tr>
<th>Case no.</th>
<th>PCNA only</th>
<th>PCNA+AgNOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>D761</td>
<td>56.7</td>
<td>54.9</td>
</tr>
<tr>
<td>D762</td>
<td>71.3</td>
<td>65.9</td>
</tr>
<tr>
<td>D765</td>
<td>43.2</td>
<td>37.2</td>
</tr>
<tr>
<td>D773</td>
<td>45.1</td>
<td>40.4</td>
</tr>
<tr>
<td>D774</td>
<td>59.7</td>
<td>54.4</td>
</tr>
</tbody>
</table>

4) Comparison of the AgNOR number in PCNA positive cells with that in PCNA negative cells

In the 12 patients with colorectal cancer, the average AgNOR number per nucleus determined for PCNA positive cells was compared with that for PCNA negative cells. AgNORs were observed as black particles in the nuclei while the PCNA positive nuclei were stained a clear red (Fig. 1).

The values of LI thus determined ranged from 24.8 to 82.7%. The average numbers of AgNORs per nucleus in PCNA positive cells ranged from 8.9 to 17.9 while those for PCNA negative cells, from 1.7 to 6.3. The results are summarized in Table 4.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>%PCNA+cells</th>
<th>Mean AgNORs/ nuclear profile in PCNA + cells</th>
<th>Mean AgNORs/ nuclear profile in PCNA–cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>D336</td>
<td>59.9</td>
<td>13.3</td>
<td>3.8</td>
</tr>
<tr>
<td>D358</td>
<td>41.9</td>
<td>9.2</td>
<td>2.6</td>
</tr>
<tr>
<td>D360</td>
<td>27.9</td>
<td>9.9</td>
<td>2.5</td>
</tr>
<tr>
<td>D362</td>
<td>63.4</td>
<td>10.9</td>
<td>3.2</td>
</tr>
<tr>
<td>D372</td>
<td>80.9</td>
<td>8.9</td>
<td>1.7</td>
</tr>
<tr>
<td>D380</td>
<td>36.2</td>
<td>9.3</td>
<td>2.9</td>
</tr>
<tr>
<td>D392</td>
<td>70.1</td>
<td>11.0</td>
<td>3.9</td>
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<tr>
<td>D399</td>
<td>24.8</td>
<td>17.9</td>
<td>6.3</td>
</tr>
<tr>
<td>D445</td>
<td>78.3</td>
<td>14.3</td>
<td>4.6</td>
</tr>
<tr>
<td>D521</td>
<td>69.6</td>
<td>12.4</td>
<td>2.2</td>
</tr>
<tr>
<td>D582</td>
<td>66.2</td>
<td>12.6</td>
<td>3.2</td>
</tr>
<tr>
<td>D593</td>
<td>82.7</td>
<td>13.6</td>
<td>3.3</td>
</tr>
</tbody>
</table>

When tested by Wilcoxon's test, the average number of 11.9 AgNORs per nucleus for PCNA positive cells was significantly greater than 3.4 for PCNA negative cells (p < 0.0001).
5) Comparison of the AgNOR number in PCNA positive cells with that in PCNA negative cells in the 12 patients with Dukes C of colorectal cancer in terms of the survival time.

In the 12 patients with Dukes C of colorectal cancer, who included dead cases with in 5 years after surgery in 6 with poor prognosis and survived over 5 years after surgery in the other 6 with fair prognosis, biologic behavior was compared by parameters of DNA index (DI), PCNA labeling index and the analysis in double staining was made in comparison with the AgNOR number in PCNA positive cells and of PCNA negative cells as shown in Table 5.

DI represented their prognoses in patients with Dukes C of colorectal cancer. Over 1.5 of DI showed poor prognosis. In contrast, 1.0 of DI indicated fair prognosis. The results of PCNA did not necessarily reflect their prognoses. However, the results of double staining of PCNA and AgNOR correlated more closely with their prognosis for patients with Dukes C of colorectal cancer. More than 10 of AgNOR number in positive PCNA cells implied poor prognosis and less than 4 of AgNOR in negative PCNA cells meant fair prognosis.

### Table 5. Significance of the results of double staining of PCNA and AgNOR in terms of the survival time in the 12 patients with Dukes C of colorectal cancer.

<table>
<thead>
<tr>
<th></th>
<th>DI</th>
<th>PCNA lobelling index</th>
<th>PCNA(+) AgNOR</th>
<th>PCNA(-) AgNOR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0&lt;1.5&lt;1.5</td>
<td>&lt; 50 &lt;</td>
<td>&lt; 10 &lt;</td>
<td>&lt; 4 &lt;</td>
</tr>
<tr>
<td>Survivor more than 5 years after surgery</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Dead within 5 years after surgery</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Discussion

NORs are the looped structures of rDNA occurring in chromosomes Nos, 13, 14, 15, 21 and 22. The rDNA is
tumor cells may be assessed more accurately. If the double-
PCNA, and AgNORs occurring in the cells with PCNA can
be counted, the proliferative activity and malignancy of
examined. If a section of tumor tissue can be stained for
site of counting even if the same preparation would be
average number per nucleus calculated after examining 100
significantly greater than the latter (p < 0.0001), indicating
PCNA negative cells was 3.4. The former number was
nuclei since the number may vary widely depending on the

method can also reflect the proliferating activity of cells.
Janmohamed et al. used the double staining method to
method in frozen and paraffin-embedded sections. In 1990,
Crocker et al. showed that the AGNOR number in low-grade
lymphomas was significantly different from that in high-grade
lymphomas. Since then, many researchers have used this
method to study various pathologic lesions, in particular
malignant tumors. These researchers have reported that the
number, size and intranuclear distribution of AGNORs
during the interphase were associated with cellular metab-
olism, multiplication and differentiation. The AGNOR num-
bearer is usually expressed in terms of the number per
nucleus, but many other units and methods to stain them
have been used.

PCNA is a non-histone nuclear protein called cyclin,
which has a molecular weight of 36kD and an isoelectric
point of 4.9. This protein accumulates in the nuclei of cells
during the late G1 to S phase of the cell cycle. It has been
known to function as a auxiliary protein of DNA poly-
merase δ. In recent years, it has been increasingly
attracting attention as an immunohistochemical marker of
cell proliferation because anti-PCNA antibody which can
be used to stain paraffin-embedded tissues for PCNA has
recently become commercially available.

In 1989, Murray et al. reported that AGNORs and
various antigens can be stained by a double staining
method in frozen and paraffin-embedded sections. In 1990,
Janmohamed et al. used the double staining method to
visualize AGNORs and Ki67 in non-Hodgkin’s lymphomas
and reported that significantly more AGNORs were con-
tained in Ki67 positive cells than in Ki67 negative cells.

No study has yet reported double-staining of AGNORs
and PCNA. The author in this study attempted to determine
optimal conditions for the sequential demonstration.

Among the 12 patients with colorectal cancer, the aver-
age AGNOR number determined by the double staining
method for the PCNA positive cells was 11.9, while that for
PCNA negative cells was 3.4. The former number was
significantly greater than the latter (p < 0.0001), indicating
that the AGNOR number determined by the double staining
method can also reflect the proliferating activity of cells.

Usually the AGNOR number is given in terms of the
average number per nucleus calculated after examining 100
nuclei since the number may vary widely depending on the
site of counting even if the same preparation would be
examined. If a section of tumor tissue can be stained for
PCNA, and AGNORs occurring in the cells with PCNA can
be counted, the proliferative activity and malignancy of
tumor cells may be assessed more accurately. If the double-
staining method had been used to stain various antigens
with AGNORs, the AGNOR number for cells of a particular
type has been determined. Consequently, this double stain-
ing method will be of value in analyzing cell kinetics.

In spite of a few patients with Dukes C of colorectal
cancer, proliferating activity of colorectal cancer cells in
Dukes C was evaluated by double staining of PCNA and
AGNOR in terms of the survival time. On the basis of the
results of this study, more than 10 AGNOR number in
PCNA positive cells is one of the borderlines in assessing
poor prognosis. In contrast, less than 4 AGNOR number in
PCNA negative cells is one of the limit in expecting fair
prognosis. However, more accumulated data should be
necessary for accurate assessment of the prognosis.

An attempt to gauge outcome in patients with colorectal
cancer has been made by histologic findings such as histo-
logic grade, pattern and inflammatory cell response, and
Dukes classification. Needless to say, the presence of
lymphatic spread and venous invasion has also become
an important criterion in evaluating the outcome. Nucleolar
organizer regions are loops of DNA (rDNA) encoded for
ribosomal RNA (rRNA) production and have been
utilized by cytogeneticists for the evaluation of a variety of
genetic disorders. NORs are estimated by so-called AG-
NOR technique on the basis of the fact that their associated
protein is argyrophilia. It is well recognized that increased
numbers of AGNOR are associated with differentiation and
poorer prognosis. However, this fact has not been uni-
formly observed.

Double staining method in combination with PCNA is
established in this study for more accurate assessment of a
given patient’s outcome. In the aspect of techniques, atten-
tion should be paid to the fact that prolonged staining will
blur the details of individual NOR and contribute to errors
of counting as recommended by Smith and Crocker. To
make up for the disadvantage of this technique, double
staining method of clinically useful tumor markers is of
great value in expecting the outcome. It is well known that
argyrophilic dots in colonic adenocarcinoma are greater
than those in benign tumors as pointed out by Derenzini et
al.

On the other hand, proliferating cell nuclear antigen
(PCNA) is a useful criterion for predicting biological
behavior in malignant tumors. It is known that PCNA is a
36 KiloDalton nuclear protein present in the nucleus of
proliferating cells which is essential for DNA replication.
PCNA has been shown to be increased in amounts in
malignant tumors but there is a less certain correlation in
some tumors.

It is sure that double staining of two parameters which
represent biological behavior is able to know the patient’s
prognosis more accurately.
References