The AgNOR count is closely related to cell proliferative activity and analysis of the DNA content by image or flow cytometry is a reliable method of predicting the prognosis of patients with various cancers. In the present study, we retrospectively examined the relationship between the AgNOR count and the DNA content in 14 patients (7 males and 7 females) with surgically resected bile duct cancer. The AgNOR count and AgNOR area were examined by a silver staining technique and the mean nuclear AgNOR count or area for at least 200 tumor cells in each specimen was calculated using the CAS 200 Image Analysis System (Cell Analysis System, Inc., Lombard, IL). DNA analysis, all specimens were done using the Quantitative Ploidy Analysis Program in the CAS 200 Image Analysis System.

The DNA ploidy pattern and diploid rate (2c rate) of 200 tumor cells were calculated and the percentage of cells with an aneuploid nuclear DNA content over 4c (4c+ rate) was also calculated. The AgNOR count and tumor cell area of aneuploid tumors were significantly (P<0.05) higher than those of diploid tumors. In addition, there was a relationship between the AgNOR count and the 4c+ rate.

In conclusion, analysis of the DNA ploidy and AgNOR parameters may provide important prognostic information in bile duct cancer.

Key words: bile duct cancer, DNA content, 4c+ rate, AgNOR count and area, malignant potential

Although the incidence of bile duct cancer is reported to be decreasing, the disease still represents a major cause of death worldwide. Despite recent improvement in surgical and multidisciplinary treatment, the prognosis for patients with bile duct cancer remains poor. Lymph node metastasis, liver metastasis, vascular involvement, and pancreatic invasion are considered to be the major prognostic factors after attempted curative resection of bile duct cancer.

Recently it is reported that tumor cell DNA content of many gastrointestinal tumors reflects the malignant and that analysis of the DNA distribution by flow cytometry (FCM) or image cytometry (ICM) is useful for predicting the prognosis. Argyrophilic nucleolar organizer regions (AgNOR) may be related to the B23 and C23 phosphoproteins of RNA polymerase I and have a regulatory function in controlling the ribosomal RNA gene transcription. The nuclear AgNOR count is directly related to the cell proliferation rate of a tumor. Our previous work has shown that the AgNOR count has a close relationship with various histopathologic factors and is of prognostic relevance in gastric cancer.

Thus, both the nuclear DNA content and the AgNOR count may be reliable prognostic indicators.

In the present study, the nuclear DNA content of tumor cells was assessed by ICM and the nuclear AgNOR count and AgNOR area were determined by image analysis. The main aim of this study was to examine whether there was a relationship between the nuclear DNA content and the AgNOR count or AgNOR area in bile duct cancer.

**Patients and methods**

The investigation was performed on histologic paraffin-embedded tumor material obtained from 14 patients (7 men and 7 women with a mean age 53.6 ± 6.7 years) who underwent resection of bile duct cancer. Two tumors were located in the upper one-third of the bile duct, three tumors were located in the mid portion, and nine tumors were located in the lower third. For DNA analysis, the tumor area within the paraffin-embedded material was identified by a pathologist using hematoxylin and eosin-stained reference sections. The 4 μm sections were cut, dewaxed, and rehydrated.

Next, the sections were stained with Feulgen using a quantitative DNA staining kit (Cell Analysis System, Inc., Elmhurst, IL.), a commercially available stain and reagent system. The sections were studied using the Quantitative Ploidy Analysis Program in the CAS 200 Image Analysis System (Cell Analysis System, Inc., Lombard, IL). At least 200 well-preserved nuclei detected in a systematic screening
of each cancer were analyzed and DNA histograms were generated for each cell population. As a control, the DNA content of normal cells in the G0/G1 phase was defined as 7.18 pg using a DNA calibration slide supplied with the CAS 200 system. The DNA ploidy pattern and the diploid rate (2c rate) of the neoplastic cells were calculated, and the percentage of cells with an aneuploid nuclear DNA content over 4c (4c+rate) was also calculated.

For assessment of the nuclear AgNOR count in tumor cells, staining was carried out according to the method of Ploton et al.\(^7\). Briefly, 4µm sections were cut from formalin-fixed, paraffin-embedded blocks, dewaxed in xylene, and rehydrated through a decreasing ethanol series to deionized water. The silver colloid solution for AgNOR staining was prepared by dissolving gelatin in 1% aqueous formic acid at a concentration of 2%, followed by mixing in a ratio of 1:2 (V/V) with 50 g/dl aqueous silver nitrate solution. Then, the sections were incubated in the dark with this solution for 40 min at room temperature, after which the colloid was washed off with distilled, deionized water, and sections were treated with 5% hyposulfate for 5 min for permanent fixation.

Subsequently, each section was dehydrated through an ethanol series to xylene and mounted in a synthetic medium.

Slides were studied using the Cell Measurement Program in the CAS 200 Image Analyzer System, with at least 200 neoplastic cells being randomly selected and evaluated for each specimen. The mean AgNOR number and mean AgNOR area per cell (AgNOR count and AgNOR area) were calculated. All data were expressed as the mean ± standard deviation. Statistical analysis was performed using Student's t-test of Spearman's rank correlation and P<0.05 was considered statistically significant.

**Results**

**DNA ploidy and AgNOR count**

There were seven diploid and seven aneuploid tumors. The AgNOR count of the aneuploid tumors was significantly higher than that of the diploid tumors (2.29 ± 0.84 vs. 3.62 ± 0.64, P<0.01). The AgNOR area of the aneuploid tumors was also significantly greater than that of the diploid tumors (3.40 ± 0.65 vs. 4.15 ± 0.54, P<0.05) (Table 1).

**2c rate and AgNOR count**

There was no relationship between the 2c rate and the nuclear AgNOR count of tumor cells (y = 80.201 - 6.7975x, r = 0.424, P = 0.131) (Fig. 1).

**4c + rate AgNOR count**

There was a weak correlation between the 4c + rate and the nuclear AgNOR count of tumor cells (y = 0.23213 + 2.8259x, r = 0.472, P = 0.088) (Fig. 2).

**Discussion**

In most human malignancies, there is a relationship between the cytometrically assessed nuclear DNA pattern of the tumor cells and the length of survival\(^8\). When neoplastic

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**Table 1** The AgNOR count and AgNOR area of the aneuploid group were significantly (P<0.01 and P<0.05) higher than those of the diploid group.

<table>
<thead>
<tr>
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<th>Diploid pattern (n=7)</th>
<th>Aneuploid pattern (n=7)</th>
</tr>
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<tbody>
<tr>
<td>AgNOR count</td>
<td>2.29 ± 0.84(^*)</td>
<td>3.62 ± 0.64</td>
</tr>
<tr>
<td>AgNOR area (µm²)</td>
<td>3.40 ± 0.65(^*)</td>
<td>4.15 ± 0.54</td>
</tr>
</tbody>
</table>

\(^*\) P<0.01, \(^*\) P<0.05
cells have an aneuploid DNA pattern, the disease is usually aggressive with rapid progression and a fatal outcome. In contrast, tumors with a diploid DNA pattern are usually less aggressive, and the patient may live a long time even with metastases. Adenocarcinoma of the bile duct ranks among the aggressive malignancies. During the last five decades, the incidence of this tumor has increased significantly in several parts of the world. However, there have been few studies of the nuclear DNA content of the cancer cells in primary adenocarcinoma of the bile duct. For this reason, DNA assessments was done by image cytometry (ICM) in the present study.

AgNOR granules are seen during metaphase in the acrocentric chromosomes 13, 14, 15, 21, and 22 in humans and have a regulatory role in the transcription of genes for ribosomal RNA. The AgNOR count is related to cell proliferative activity and increases during G1 phase to reach a peak during S-phase of the cell cycle. In proliferating cells, the AgNOR count rises as the proliferative activity and it has been found to correlate with other proliferative markers such as DNA flow cytometry and the Ki-67 labelling index. However, there have been few reports on the relationship between the DNA distribution and AgNOR of bile duct cancer. Therefore, we investigated whether there was a relationship between DNA ploidy and the AgNOR count and whether these parameters could provide any prognostic information of the individual patient.

Both the AgNOR count and AgNOR area in aneuploid tumors were significantly greater than those in diploid tumors. There were no significant differences of age, surgical stage, and operative procedure between the patients with diploid and aneuploid tumors, making it reasonable to compare survival period in these 2 groups. In diploid group, no patient died within two years after surgery. On the contrary, two patients died of liver metastasis or local recurrence within two years in the aneuploid group. This suggested that both DNA ploidy pattern and AgNOR indices such as the AgNOR count and AgNOR area might be useful in predicting the prognosis of bile duct cancer, although it is difficult to draw conclusions because 14 patients is too small a number. Another observation was the weak correlation between the AgNOR count and the 4c+ rate. In previous studies on carcinoma of the breast or pancreas, tumors with triploid cells were found to be particularly aggressive. This suggested that the percentage of triploid or tetraploid cancer cells reflected the malignant potential of a tumor, so we selected the 4c + rate as a possible index for the determination of prognosis because it might reflect proliferative activity.

In patients with bile duct cancer, cytology of cells aspirated from the percutaneous transhepatic drainage tube (PTCD) is frequently done and cytodiagnosis of tumor cells in the bile juice is important in differentiating this cancer from various benign tumors. Preoperative knowledge of the aggressiveness of bile duct cancer can also contribute to selecting the appropriate operative procedure or postoperative adjuvant therapy. Thus, cytodiagnosis by preoperative DNA or AgNOR assessment may help in selecting the proper therapeutic method.

In conclusion, preoperative assessment of the DNA ploidy and AgNOR count of bile duct cancer by aspiration of bile juice may contribute to selection of the best therapeutic method, including operative procedure and adjuvant chemotherapy. However, further investigation is necessary to determine whether the AgNOR count and 4c+ rate are the strongest prognostic indicators for bile duct cancer apart from the operative procedures.

References