Flow Cytometric Analysis of the Cellular DNA Content in Various Liver Diseases

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Summary: Flow cytometric analysis of the cellular DNA content was conducted using paraffin-embedded liver tissues. Of the 488 cases examined, 413 (84.6%) cases were suitable for analysis. Of these, 296 (88.9%) were diploid and 37 (11.1%) were aneuploid. The ploidy pattern was unaffected by patients' sex and age. Long-term stored tissues were as suitable for analysis as short-term stored ones. An aneuploid pattern was more frequently found in hepatocellular carcinoma (HCC) (17/43, 39.5%) than in non-neoplastic liver conditions (20/284, 7.0%) ($p < 0.01$). The ploidy pattern was determined to be one of the most important prognostic factors in HCC. An aneuploid pattern was also found in non-neoplastic liver conditions such as liver cirrhosis (LC) and chronic hepatitis caused by hepatitis virus (CH).

It seems that the ploidy pattern and proliferative index (S+G2M fraction) do not always affect carcinogenesis in non-neoplastic liver conditions. Thus, flow cytometric analysis of the cellular DNA content using paraffin-embedded specimens is useful for determining the prognostic significance in HCC, although the significance is uncertain in non-neoplastic liver conditions.

Introduction

Flow cytometric analysis of the cellular DNA content of various solid tumors using paraffin-embedded specimens has been established[1-3]. An increasing number of reports have been published concerning the correlation between the ploidy pattern and the prognosis[4-8]. In our previous studies[9], the ploidy pattern served as a useful prognostic marker for hepatocellular carcinoma (HCC). On the other hand, reports on the ploidy pattern of non-neoplastic liver specimens and the correlation with prognosis are relatively few[10-12]. The purpose of this study is to evaluate the frequency of aneuploidy and its correlation with the prognosis, as well as to analyze the cell cycle and determine its clinical significance.

Materials and Methods

During the period from 1976 to 1989, 488 paraffin-embedded blocks obtained from liver biopsies or surgically resected liver tissues were collected at Nagasaki University Hospital, Nagasaki Municipal Medical Center and several other major hospitals in and outside Nagasaki Prefecture (Appendix). The cases consisted of 396 males and 92 females, ranging in age from 15 to 76 with an average of 49.0 ± 13.1 years.

All materials had been formalin-fixed, paraffin-embedded and stored at room temperature. For measuring the cellular DNA content, the method of Hedley et al.[1] was enployed with minor modifications. Sections were cut with a microtome to be a thickness of 50 μm. Two or three sections were required for each tissue block. The sections were de-waxed using xylene, and re-hydrated in a sequence of 100, 100, 95, 90, 85, 70, and 50% ethanol for 30min each at room temperature. The tissue was washed in distilled water and digested in 1.0% pepsin solution (Sigma, St. Louis, MO), pH 1.5, in a waterbath at 37 °C for 90min, with frequent vortex mixing. Then the tissue was filtered through a 50 μm nylon mesh and centrifuged, and the pellet was resuspended in 1mg/ml of ribonuclease (Sigma, St. Louis, MO) at 37 °C for 30min. It was centrifuged again and the pellet was resuspended in 0.1% Nonidet P-40 and then resuspended and stained with 50 μg/ml of propidium iodide (Sigma, St. Louis, MO) at room temperature in the dark for 20min.

The cellular DNA content was measured using a flow cytometer (FACScan, Becton-Dockinson, Mountain View, CA). Propidium iodide was excited with an air-cooled 15mW argon ion laser operating at 488nm, and fluorescence above 585nm was measured through a long-pass filter. Ten thousand cells were examined in each sample, and the DNA content measured by fluorescence intensity was recorded (Fig. 1). An internal DNA standard was provided by the normal diploid cells present in each sample. DNA histograms were analyzed using the FACScan computer programs CONSORT 30 and DNA Cell-Cycle Analysis Software.[13, 14]

Statistical analysis was performed using the chi-square test and Student's t test. Survival of different groups was compared using the generalized Wilcoxon test, and Cox's proportional hazard model was adopted as a multivariate model suitable for evaluation of prognostic factors. A

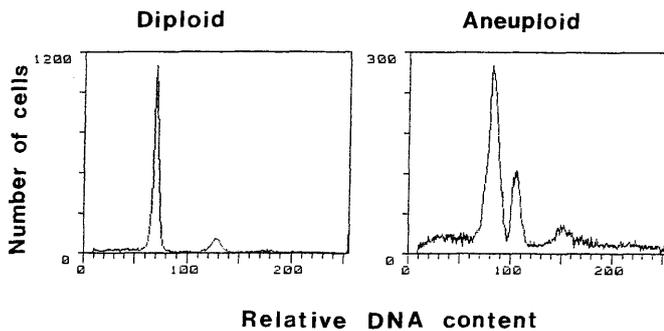p-value of less than 0.05 was considered significant.

Fig. 1. DNA histogram of diploid and aneuploid pattern.

Results

Of a total of 488 tissues, 413 (84.6%) were suitable for analysis. The average coefficient of the variation (CV) value of G0/G1 peaks in the histogram was 5.84 ± 1.54%. A CV value of less than 7.0% was observed in 77.0% of the specimens. Judgment of the ploidy pattern became difficult when the CV value was 7.0% or more. Therefore, a diploid pattern was confirmed only when the CV value was less than 7.0%. It was found that 296 (88.9%) cases were diploid and 37 (11.1%) aneuploid. There was no significant difference in the frequency of aneuploidy by sex and age. The frequency of suitability for analysis, the average CV value and the frequency of aneuploidy were unaffected by the period between biopsy or resection and measurement (Table 1).

Table 1. Correlation of the CV values with periods between biopsy or resection and measurement

Period(years)[a]	No. of cases	No. of analyzed cases[b]	CV values[c](%) Mean ± S. D.
0 – 2	88	73	5.66 ± 1.75
2 – 4	118	107	5.61 ± 1.51
4 – 6	116	103	5.92 ± 1.60
6 – 8	88	70	6.21 ± 1.48
8 – 10	44	34	5.87 ± 1.39
10 – 12	31	24	5.85 ± 1.05
12 – 14	3	2	7.05 ± 1.34
Total	488	413	5.84 ± 1.54

a: period between biopsy or resection and measurement (years).
b: cases suitable for analysis.
c: coefficient of variation.

Histologically definite diagnosis was made for 290 diploid cases of 296, and all 37 aneuploid cases. Of these 327 cases, 43 were HCC, 73 liver cirrhosis (LC) without HCC (LC without HCC), 37 LC with HCC, 107 chronic hepatitis (CH) without HCC (CH without HCC), 10 CH with HCC, 37 alcoholic hepatic fibrosis, 5 subsiding stage of acute hepatitis, 2 fatty liver, and 13 histologically normal liver

tissue. In LC with HCC and CH with HCC, specimens were taken from the non-cancerous part of the liver.

The prognostic implication of the ploidy pattern in HCC was reported in the Japanese Journal of Cancer Clinics, in 1990[9]. We investigated 41 cases of HCC and found that 25 (61.0%) cases were diploid and 16 (39.0%) were aneuploid. By means of a Cox's proportional hazard model, this ploidy pattern was determined to be one of the most important prognostic factors (p=0.0116). The prognosis was worse in patients with aneuploid tumors than in those with diploid tumors (Fig. 2).
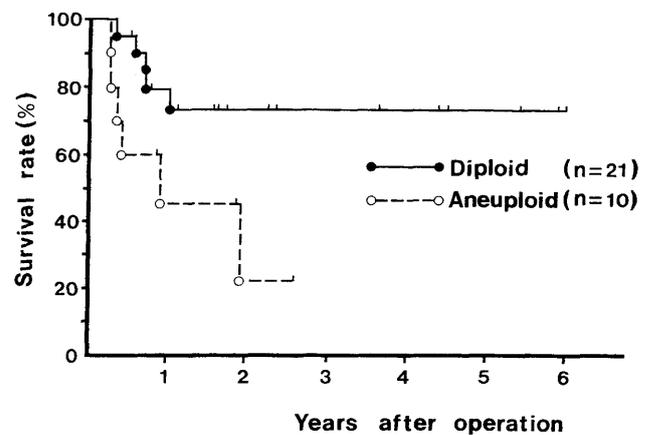

Fig. 2. Survival related to DNA ploidy pattern in hepatocellular carcinoma (Kaplan-Meier's survival curve). The survival rate of aneuploid cases was significantly lower than that of diploid ones (p < 0.05).

An aneuploid pattern was more frequently found in HCC than in non-neoplastic liver conditions (p < 0.01) (Table 2). Aneuploidy was found mainly in HCC, LC and CH, and was not found in acute hepatitis, fatty liver, or normal liver. In alcoholic hepatic fibrosis, only one case revealed aneuploid pattern. Classified by etiology, aneuploidy was more frequently found in type B chronic liver disease than in non-A non-B chronic liver disease and alcoholic liver disease, but this difference was not significant (p > 0.05) (Table 3). Among 9 cases of aneuploidy in non-neoplastic liver conditions without HCC that were followed up for over one year after the biopsy, HCC was detected in only one case, 76 months later.

The distribution of cell fractions in the G1, S, and G2+ (M) phases was analyzed for 259 diploid cases with suitable DNA histograms, that is, less than 7.0% in CV value and less than 3.0 in weighted variance. (The weighted variance is a statistic method to estimate how well the model fits the data. The number to reflect the best fit is 1.) The S + G2 (M) fraction value was higher in HCC and LC with HCC than in LC without HCC (p < 0.01). This value was also higher in CH with HCC than in CH without HCC (p < 0.01) (Fig. 3). Among 17 cases without HCC showing a high S + G2 (M) fraction value (higher than the average

Table 2. Comparison in ploidy pattern between hepatocellular carcinoma and non-neoplastic liver conditions

Disease groups	No. of cases	Ploidy pattern		
		Diploid	Aneuploid	(%)
HCC[a]	43	26	17	(39.5)[c]
Non-neoplastic [b] liver conditions	284	264	20	(7.0)[c]

a: hepatocellular carcinoma. b: including liver cirrhosis (with and without hepatocellular carcinoma), chronic hepatitis (with and without hepatocellular carcinoma), alcoholic liver fibrosis, acute hepatitis, fatty liver, and normal liver. c: p < 0.01

Table 3. Ploidy pattern in non-neoplastic liver conditions

Disease groups	No. of cases	Ploidy pattern		
		Diploid	Aneuploid	(%)
LC without HCC	73	67	6	(8.2)
Alcoholic	36	33	3	(8.3)
Type B	19	17	2	(10.5)
Non-A, non-B	18	17	1	(5.6)
LC with HCC	37	34	3	(8.1)
CH without HCC	107	97	10	(9.3)
Type B	43	37	6	(14.0)
Non-A, non-B	64	60	4	(6.3)
CH with HCC	10	10	0	(0.0)
Alcoholic liver fibrosis	37	36	1	(2.7)
Acute hepatitis	5	5	0	(0.0)
Fatty liver	2	2	0	(0.0)
Normal liver	13	13	0	(0.0)

LC: liver cirrhosis.   HCC: hepatocellular carcinoma.
CH: chronic hepatitis.
In LC with HCC and CH with HCC, specimens were taken from the non-cancerous part of the liver.

Fig. 3. Proliferative index in various liver diseases. This value was higher in HCC and LC with HCC than in LC without HCC, and also in CH with HCC than in CH without HCC.
HCC: hepatocellular carcinoma. LC: liver cirrhosis.
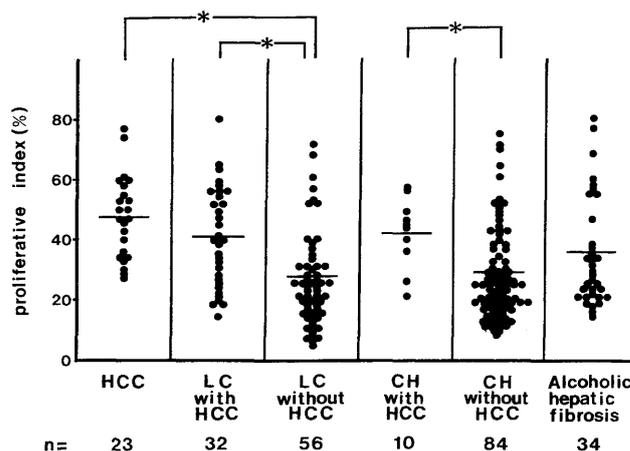CH: chronic hepatitis.
In LC with HCC and CH with HCC, specimens were taken from the non-cancerous part of the liver.
* :p<0.01

value in the same disease with HCC), that were followed up for over one year after the biopsy, there was no case that developed HCC.

Discussion

Measurement of the cellular DNA content by flow cytometry used to require fresh unfixed tissue as a starting material. Since Hedley's report in 1983[1], the use of paraffin-embedded specimens became possible for this type of study. In this study, 84.6% of materials were suitable for analysis. Furthermore, the result was unaffected by the period between biopsy or resection and measurement. Therefore, long-term stored paraffin-embedded liver tissues are also suitable for analysis as long as they have been embedded in an ordinary manner. This method therefore allows the retrospective study of paraffin-embedded specimens. A few reports have been published concerning the correlation between the ploidy pattern and the prognosis in HCC using this method[8,9].

It is especially noteworthy that aneuploidy was found not only in HCC but also in non-neoplastic liver conditions.

Roncalli et al.[12] reported that liver-cell dysplasia is a heterogeneous lesion in terms of ploidy, but a few reports indicate the presence of aneuploidy in non-neoplastic liver conditions using microspectrophotometry or flow cytometry[10,11,15]. Mito et al.[15] reported that aneuploidy was found in LC, but not in normal liver tissue and CH. We found aneuploidy also in CH and alcoholic hepatic fibrosis, although only one aneuploidy case was found in the latter. Therefore, it is suggested that the ploidy pattern in CH is affected by hepatitis virus, seemingly more by type B hepatitis virus than by non-A non-B hepatitis virus. The significance of this result is uncertain. This may suggest that hepatitis virus affects carcinogenesis. However, it does not seem that aneuploidy always supports its pre-neoplastic nature in non-neoplastic liver conditions.

Flow cytometric analysis of the cell cycle using paraffin-embedded specimens presents many problems. In our method, the presence of cellular debris and quiescent cells can cause confusion. Cellular debris is minimized at a section thickness of 50 μm^3, but quiescent cells confuse the measurement of the S-phase fraction. Ohyama et al.[16] reported that cell cycle analysis performed from DNA histogram was not reliable with respect to the S-phase fraction, and that the proliferative index (S + G2M fraction) should be regarded as a proliferative activity in one parameter analysis. We measured the same specimens of 3 cases several times, and the result of the proliferative index, though variable, showed the same trend individually (Fig. 4). Therefore, evaluation of the absolute value of the proliferative index is unreliable, but comparison of their relative values seems meaningful to some degree. As a result, it is suggested that DNA synthesis is more accel-
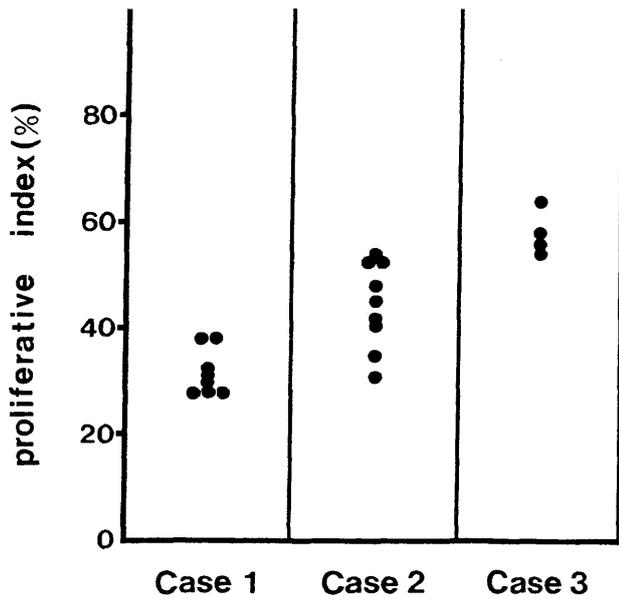
Fig. 4. Proliferative index measured several times individually in three cases.

erated in non-neoplastic tissues with HCC than in those without HCC. Tarao et al.[17] reported that cirrhotics with high DNA synthetic potency stood a high risk of developing HCC. However, our results suggest that a high value of the proliferative index does not always support its pre-neoplastic nature in our method.

In conclusion, flow cytometric analysis of the DNA content using paraffin-embedded liver tissues is useful for determining the prognostic significance in HCC, but many problems are present in non-neoplastic liver conditions. More data will be needed before the clinical implications of these findings become evident.

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Appendix

Institutions wherefrom blocks were collected:
Second Department of Internal Medicine, First and Second Department of Surgery, Nagasaki University Hospital
Nagasaki Municipal Medical Center
Sasebo Municipal General Hospital
National Sanatorium Higashi-Saga Hospital
Kitakyushu City Yahata Hospital
Shunkai-kai Inoue Hospital
Isahaya Insurance General Hospital
Goto Central Hospital
Yuhaku-kai Senju Hospital
Nagasaki Prefectural Shimabara Onsen Hospital