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# Evaluation of Prognosis for Borrmann Ⅳ Gastric Cancer
## —— In Terms of DNA Analysis and Nucleolus Number ——

Hideo CHIJIWA

*The First Department of Surgery,*
*Nagasaki University School of Medicine*

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**ABSTRACT :** It is well known that Borrmann Ⅳ gastric cancer allows a most formidable prognosis. The influential factors on the prognosis of the 40 patients with Borrmann Ⅳ gastric cancer were evaluated by means of DNA analysis and nucleolus assessment of neoplastic cells obtained from embedded block of surgical specimens. The prognosis in patients with aneuploid pattern which account for 77.5% in this series was worse rather than that with diploid pattern on DNA histogram and also histologic findings of vascular invasion of ly (+), v (+) were mostly included into aneuploid pattern. It is assumed that the smaller the size of nucleolus in diameter, the higher the incidence of aneuploid pattern and the worse the prognosis of Borrmann Ⅳ gastric cancer has become.

It is concluded that combination of DNA analysis with assessment of nucleolar number and size is required for detailed evaluation of the prognosis for Borrmann Ⅳ gastric cancer.

## INTRODUCTION

With a prevalence of mass survey for early detection of carcinomas in various organs, surgery for early gastric cancer is now increasing. In contrast, surgery for advanced gastric cancer of Borrmann Ⅳ still accounts for 10% or more and remains unchanged in frequency[1]. It is well known that gastric cancers of Borrmann Ⅳ were characteristic of rapid spreading in spite of symptomatically silent phase.

The purpose of this study is to clarify cell-proliferation and nuclear activity of Borrmann Ⅳ cancer cells from the standpoint of DNA and nucleolus analyses and to define the correlation between clinicopathological factors and prognoses.

## MATERIAL AND METHOD

Surgical specimens obtained from 40 gastic cancers were examined and also obtained from the histopathologic files of First Department of Surgery, Nagasaki University School of Medicine during the time from January 1983 to December 1987.

Single cells with denuded nuclei were prepard as described by Shutte[2] as follows (**Fig. 1**). Sections were cut at 50$\mu$m thickness from routinely processed paraffin blocks. These were dewaxed in a sequence of xylen, then dehydrated through a graded series of ethanol to deionized water. This procedure was repeated twice a hour. Thereafter, the tissue was incubated overnight at 37°C with 3mM trisodium citrate, 0.1% Nonidet P40, 1.5mM spermine tetrahydrichloride, 0.5mM trisbuffer pH7.6, filtrated with 50$\mu$m nylon mesh.

Propidium Iodide (PI) staining accorded with

Materials & Methods (Schutte, B. R., 1985)

Paraffin-embedded material
50$\mu$m sections
↓
dewaxed (xylene)
rehydrated (100%, 95%, 70%, 50% ethanol)
washed in distilled water
↓
Trypsin-citrate buffer
incubated overnight at 37°C
↓
staining
solution A (Trypsin)
solution B (Trypsin inhibiter, RNase A)
solution C (spermine tetrahydrochrolide
Propidium lodide)
↓
fluorescence measurement
FACS IV
DNA Index = peak channel No. of cancer cells / peak channel No. of normal cells

**Fig. 1.** Measurement steps.

the method as reported by Vindelφv and associates[3]. Sections were cut at 3$\mu$m thickness. These were dewaxed in xylene, then dehydrated through ethanol (100, 95, 70, 50%) for 30min, to deionized water for one hour, incubated overnight with 3mM trisodium citrate, 0.1% Nonidet P40, 1.5mM spermine tetrahydrochlorid, 0.5mM trisbuffer pH7.6 and filtrated with 50$\mu$m nylon mesh and free cells with denuded nuclei were prepared as the above procedures.

PI staining was made by adding A solultion (Trypsin 15mg, Stock Solution 500ml) to 200ml cell suspension and left for 10min at room temperature, then B solution (trypsin inhibitor 250mg, RNase A 50mg, Stock Solution 500ml) was added and left for 10min, thereafter 1.5ml C solution (PI 250mg, Spermine tetraphdrochloride 580mg, stock solution 500ml) was added and kept in ice for 30min. For flow cytometric analysis, FACS IV was used for this study. DNA histogram was delineated by using argon laser with 580mm filter.

DNA index (DI) was calculated as the following formula (peak channel in $G_0G_1$ phase of tumor cells/peak channel in lymphocytes around the tumor). DI of 1.0 was named diploidy and DI except for 1.0 was called aneuploidy including a case of the peak in 4C and the presence of $G_2M$ phase in 8C on DNA histogram. The result that showed coefficients of variation (CV) of more than 10 was excluded from this study and reexamined in this study.

The results were analyzed in accordance with $X^2$ test and Kaplan-Meier method. The assessments of cancer cell nucleolus were made by counting 100 cancer cells at random under ×100 magnifying light microscopy using HEstaining preparats. The producibility was kept by taking over 10 photograms each preparat.

The sizes of nucleous were expressed as large, more than 2.0$\mu$m in diameter and as small, less than 2.0$\mu$m. **Fig. 2** showed small nucleolus in the majority and **Fig. 3** represented large nucleolus in most. A statistically significant

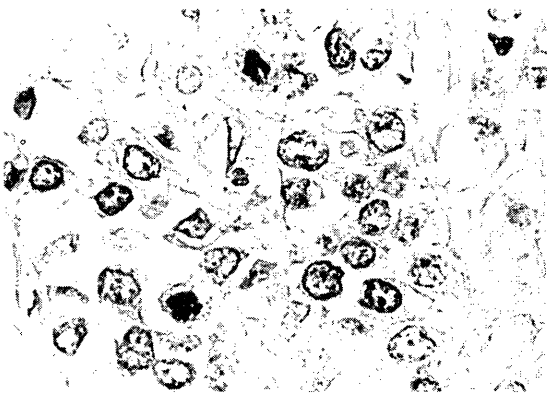

**Fig. 2.** Microscopic finding shows that gastric cancer cells with small nucleoli are predominant.

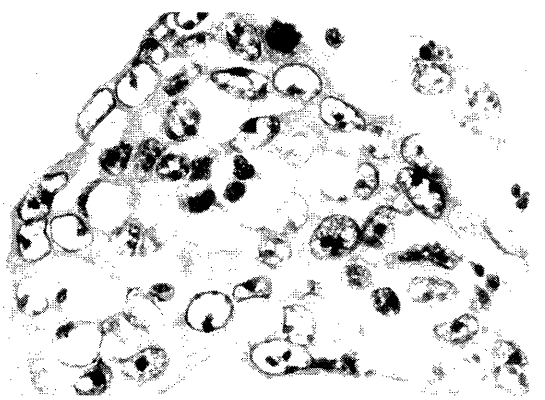

**Fig. 3.** Microscopic finding shows that gastric cancer cells with large nucleoli are predominant.

difference was regarded as the value of less than 0.05. As for histopathological analysis, the expression regarding histopathology accorded with the general rules for the gastric cancer study in surgery and pathology (Japanese Research Society for Gastric Cancer).

## RESULTS

**Table 1** showed DNA histogram obtained from the specimens of the 40 patients with gastric cancer. Aneuploid patterns of **Figure 4a** on the DNA histogram was obtained from normal cells and that of **Figure 4b** was from tumor cells in which DI was 1.45 and CV was 3.9% respectively. DNA ploidy patterns displayed disploid in 9 (22.5%) and aneuploid in 31 (77.5%) (Table 1).

The average age was 54.4±11.5 in diploid group and 59.4±12.6 in aneuploid group. Sex distribution of men to women was 21 : 19. Men were somewhat predominant. According to disease stage, most of aneuploid patterns inclueded gastric cancers of Stage III and IV. As for the depth of cancer infiltration, two cases with no infiltration to the serosal layer, PS (−), showed aneuploid pattern. In contrast, thirty-eight with PS (+) included eight with diploid pattern and 30 with aneuploid (**Table 2**).

According to histologic findings, nine showed well differentiation in whom one was diploid and eight were aneuplid. On the other hand, 31 revealed poorly differentiation in whom eight were diploid and 23 aneuploid (**Table 2**). With respect to INF classification, there was no patient with pattern of INF$\alpha$. The nine patients with INF$\beta$ were divided into one with diploid patterns and eight with aneuploid. On the contrary, the 31 patients with INF$\gamma$ included eight with diploidy and 23 with aneuploidy (**Table 3**). It was a similar pattern with histologic types.

Accroding to the classification of lymphatic invasion (ly), the five patients with ly (−) showed only diploid pattern. On the other hand, the 35 patients with ly (+) included four patients with dipolidy and 31 with aneuploidy (**Table**

**Table 1.** Relationship between DNA ploidy pattern and age, sex, or stage of Borrmann IV gastric cancer.

| | | n | Diploidy (%) | Aneuploidy (%) |
|---|---|---|---|---|
| Age (mean) | | 58.3±12.4 | 54.4±11.5 | 59.4±12.6 |
| Sex | M | 21 | 1 ( 5) | 20 (95) |
| | F | 19 | 8 (42) | 11 (58) |
| Stage | I, II | 0 | 0 | 0 |
| | III | 11 | 3 (27) | 8 (73) |
| | IV | 29 | 6 (21) | 23 (79) |
| Total | | 40 | 9 (22) | 31 (78) |

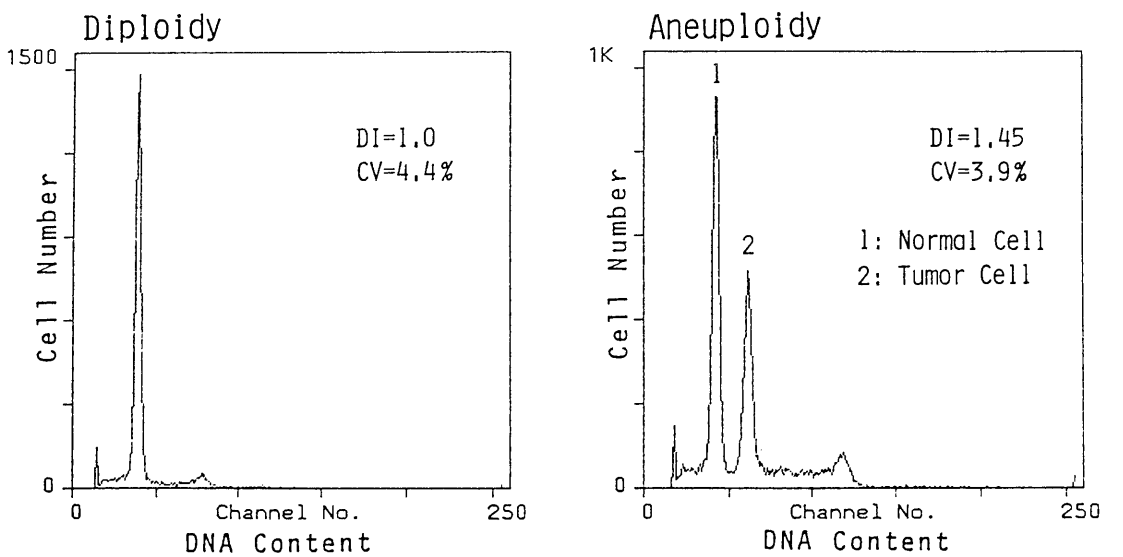


**Fig. 4.** DNA histograms of normal cells (a, left) and cancer cells (b, right).

**Table 2.** Relationship between DNA ploidy pattern and depth of the invasion and histology.

| | | | Diploidy (%) | Aneuploidy (%) |
|---|---|---|---|---|
| Depth of the invasion | ps(−) | 2 | 0( 0) | 2(100) |
| | ps(+) | 38 | 8(21) | 30( 79) |
| Histology | | | | |
| Well diff, adenoca, | | 9 | 1(11) | 8( 89) |
| Poorly diff, adenoca, | | 31 | 8(26) | 23( 74) |
| | | 40 | 9(22) | 31( 78) |

**Table 3.** Relationship DNA ploidy pattern and INF classification lymphatic invasion or vascular invasion.

| | | Diploidy (%) | Aneuploidy (%) | |
|---|---|---|---|---|
| INF α | 0 | 0 | 0 | |
| β | 9 | 1( 11) | 8(89) | |
| γ | 31 | 8( 26) | 23(74) | |
| ly(−) | 5 | 5(100) | 0( 0)— | * |
| (+) | 35 | 4( 11) | 31(89)— | |
| v(−) | 15 | 7( 47) | 8(53)— | * |
| (+) | 25 | 2( 8) | 23(92)— | |
| | 40 | 9( 22) | 31(78) | |

Chi square test: *$p<0.01$

3). It was statistically different ($p<0.01$) in aneuploid pattern between patients with ly(−) and ly(+).

As for classification of histologic findings of vascular invasion (v), the 15 patients with v(−) included seven with diploidy and eight with aneuploidy. On the other hand, the 25 patients with v(+) were divided into two with diploidy and 23 with aneuploidy (**Table 3**).

The statistical analysis for patients with aneuploid pattern revealed a significant difference ($p<0.01$) between patients with v(−) and v(+).

Concerning nodal involvement, diploid showed in two and aneuploid in one in the three patients without nodal involvement. On the other hand, in the 37 patients with positive node metastasis, seven patients showed diploid and 30 were aneuploid. There was a statistical significance ($p<0.05$) in aneuploid pattern on DNA histogram between the six patients with $n_0n_1$ and 26 patients with $n_2n_3n_4$ (**Table 4**).

As for hepatic metastasis, the 37 patients had not hepatic metastasis including eight in diploidy and 29 in aneuploidy. On the other hand, the three patients with positive hepatic metastasis included one in diploidy and two in aneuploidy (**Table 5**).

According to peritoneal dissemination, p(−) patient was 18 including five in diploidy and 13 in aneuploidy. On the other hand, p(+) patients accounted for 22 in whom diploid included four patients and aneuploid comprised of 18 patients. There was not statistically significant difference.

In this series, intra-tumoral heterogeneity was

**Table 4.** Relationship between DNA ploidy pattern and lymph node involvement.

| | | Diploidy (%) | Aneuploidy (%) |
|---|---|---|---|
| n(−) | 3 | 2(67) | 1(33) |
| (+) | 37 | 7(19) | 30(81) |
| $n_0$ | 3 | 2(67) | 1(33) |
| $n_1$ | 8 | 3(38) | 5(62) |
| $n_2$ | 13 | 1( 8) | 12(92) |
| $n_3$ | 9 | 2(22) | 7(78) |
| $n_4$ | 7 | 1(14) | 6(86) |
| | 40 | 9(22) | 31(78) |

**Table 5.** Relationship DNA ploidy pattern and hepatic metastasis or peritoneal dissmination.

| | | Diploidy (%) | Aneuploidy (%) |
|---|---|---|---|
| H(−) | 37 | 8(22) | 29(78) |
| (+) | 3 | 1(33) | 2(67) |
| P(−) | 18 | 5(28) | 13(72) |
| (+) | 22 | 4(18) | 18(82) |
| | 40 | 9(22) | 31(78) |

seen in 16 (42%) out of 38. There was a statistical significance ($p<0.05$) in apperance of intra-tumoral heterogeneity between v (−) and v (+) patients and the difference between ly (−) and ly (+) patients (**Table 6**). There was no significant difference between prognosis and DNA ploidy pattern (**Fig. 5**).

**Fig. 6** showed the correlation between number and size of nucleolus of tumor cells and DNA ploidy pattern. There was a significant difference ($p<0.01$) between the number of nucleolei of the patients with diploidy (249.6±77.8) and those of the patients with aneuploidy (332.1±69.9).

As compared with the number of nucleolus, there was a significant difference ($p<0.05$) in a one year survival rate following surgery between patients with the number of nucleolus of more than 250 and less than 250 (**Fig. 7**).

In the patients with diploid pattern, there was a significant difference ($p<0.05$) in one year survival between patients with the number of nucleolus of less than and more than 250 (**Fig. 8**).

In the patients with aneuploid pattern, there was only a significant difference in the half of a year survival (**Fig. 9**). On the other hand, there was a significant difference ($p<0.05$) in % nucleolus of less than 2$\mu$m in diameter between aneuploid (90.0±11.1%) and diploid patterns (81.7±19.6%) (**Table 7**). Although there was no difference between DI and the number of nucleolus.

**Table 6.** Relationship between intra-tumoral heterogeneity and stage, histology, lymphatic invasion or vascular invasion.

| | n | (−) (%) | (+) (%) |
|---|---|---|---|
| Stage 3 | 10 | 6 ( 60) | 4 ( 40) |
| 4 | 28 | 16 ( 57) | 12 ( 43) |
| Histology | | | |
| Well Diff, adenoca, | 7 | 4 ( 57) | 3 ( 43) |
| Poorly diff, adenoca, | 31 | 18 ( 58) | 13 ( 42) |
| ly (−) | 5 | 5 (100) | 0 ( 0) |
| (+) | 33 | 17 ( 52) | 16 ( 48) |
| v (−) | 13 | 11 ( 85) | 2 ( 15) |
| (+) | 25 | 11 ( 44) | 14 ( 56) |
| Total | 38 | 22 ( 58) | 16 ( 42) |

$p<0.05$

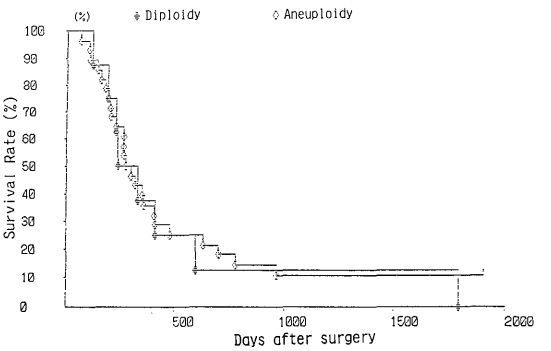


**Fig. 5.** Comparison of survival rates between the patients with diploid and those with aneuploid.

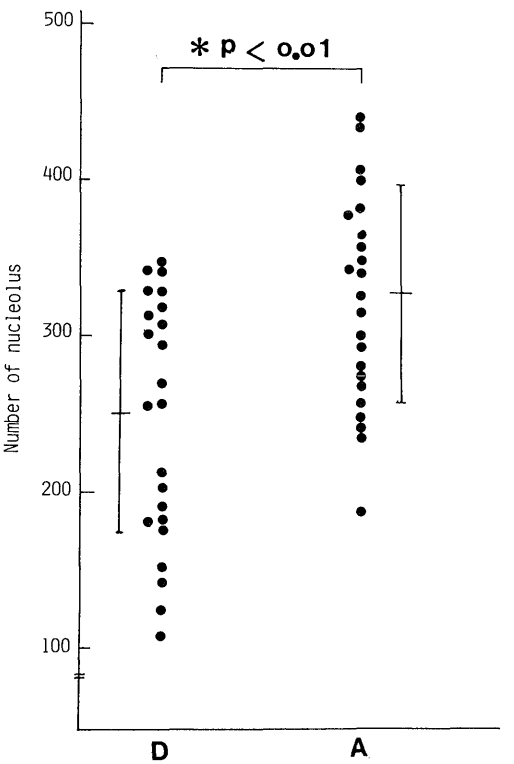


**Fig. 6.** Relationship between DNA ploidy pattern and the number of nucleolus of the cancer cells.

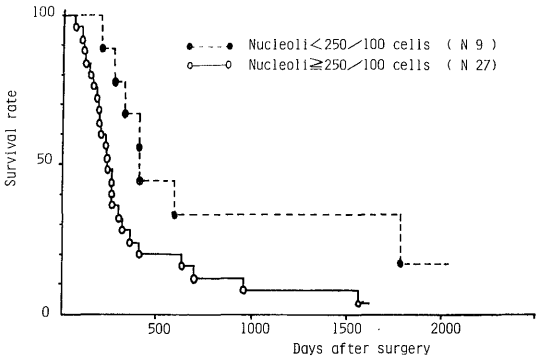


**Fig. 7.** Comparison of survival rates between the patients with nucleoli less than 250 per 100 cancer cells and those with more than 250.

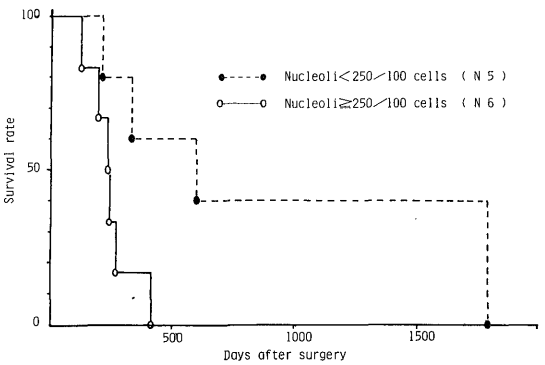


**Fig. 8.** Survival rates of the patients with diploid pattern according to the number of nucleoli.

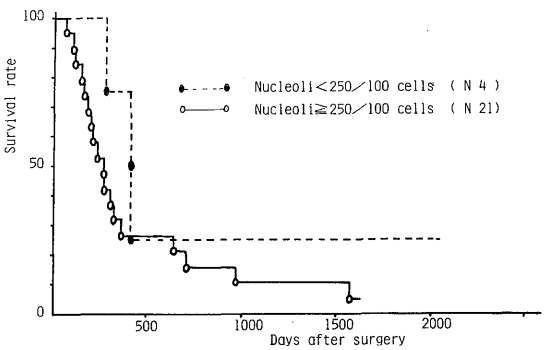


**Fig. 9.** Survival rates of the patients with aneuploid pattern according to the number of nucleoli.

**Table 7.** Percentage of nucleolus of less than 2 $\mu$m in diameter according to DNA ploidy pattern.

| Diploid | Aneuploid |
|---|---|
| 81.7±19.6 | 90.0±11.1 |

$p<0.05$

## DISCUSSION

The study on biologically aggressive behavior of malignant cells does not provide any valuable explanation. Clinical application of DNA analysis is of great benefit in part to assess the patient's prognosis and to determine the necessity of the subsequent adjuvant therapy.

Recently, DNA analysis has become a valuable tool for assessment of aggressive proliferation of malignant cells[6,7,8,9]. Flow cytometry is now beneficial in the practical aspect as a simple, quick and reproducible method.

Since Hedley[10] reported the method of DNA analysis from paraffin-embedded block in 1983, the confidence of the results obtained from this method has been substantiated[11]. As a result, DNA analysis evidenced the availability of assessing the survival time in patients with carcinomas. It is accepted that the prognosis of patients with Borrmann IV gastric cancer is very poor. Worse prognosis origninates from difficulty in early detection by routine procedures and associates with rapid cancer spreading through peritoneal dissemination.

Yonemura[12,13] reported that early gastric cancer demonstrated 40% diploid pattern and 60% aneuploid patterns. In this series, Borrmann IV gastric cancer included 34.4% diploid and 66% aneuploid patterns. In advanced gastric cancer, Matsumoto[14] clarified that 26% were diploid, 53% were in combination of diploid with aneuploid and 21% were aneuplpoid patterns. Some reported the deeper the depth of cancer infiltration, the higher the incidence of high DNA ploidy[15,16]. On the other hand, Hattori[17] and Czerniak[18] cited that nuclear DNA patterns in gastric cancer cells were definitively fixed before early gastric cancer progressed.

There is no constant relationship in the incidence of aneuploid pattern between early and advanced gastric cancers. It is suggestive of possibility of new development of aneuploid stem cell line.

In this study, advanced gastric cancers tend to display aneuploid pattern in analysis of the relationship between the factors of ly(+) v(+) and node metastasis. It is concluded that aneuploid pattern of gastric cancer cells is liable to express an aggressive proliferation of malignant cells on the basis of the results in this study.

According to the reports by Nishimura[19] and Yamamoto[20], the majority of patients with hepatic metastasis revealed aneuploid pattern although there was no significant difference in hepatic metastasis between H(+) and H(−) patients in this study. The incidences of hepatic metastasis and node metastasis increase in well-differentiated carcinomas. It is reported by Inokuchi[21] and Sano[22] that the prognosis for well differentiated carcinomas is worse rather than that for undifferentiated carcinomas. In contrast, Ebara[23] clarified that 85% of Borrmann IV gastric cancer cells revealed poorly differentiated carcinoma. Hattori[24,25] reported that gastric cancer cells of scirrhus type were characteritic of the pattern of poorly differentiated carcinoma and it is more likely to demonstrate aneuploid pattern.

Hirose[26] clarified that aneuploid cancer was rare in frequency in early stage of undifferentiated carcinoma although it increased in accordance with progression of carcinoma. On the contrary, the incidence of aneuploid cancer in well differentiated carcinomas was almost constant in accordance with progression of gastric cancer. It is argued that it is chatacteristic of Borrmann IV gastric cancer that poorly differentiated carcinoma is main in the aspect of histologic finding and the majority demonstrate aneuploid pattern on DNA histogram.

Recent studies on DNA analysis focus on heterogeneity of tumor cells[27]. The incidence of heterogeneity has been reported as being 40% by Sasaki[28], 33% by Aretxabela[29] and 63% by Matsumoto[14].

In this series, it was certained that 41% of heterogeneity was confirmed in Borrmann IV gastric cancer cells and it is more frequent in ly(+) and v(+) gastric cancer regardless of histologic types. From the above results, the author emphasized that in cases of diploid gastric cancers, repeated assessments of DNA analysis are needed for high incidence of heterogeneity.

Particular attention has been paid to the investigation of nucleolus in which ribosomal RNA is synthesized and also it is altered in relation to synthetic activity. Black[30] investigated the correlation among such factors as size, shape, staining and chromosome number in the nucleolus of neoplastic cells and the patient's prognosis. Recently it is ascertained that nucleolar organizer regions (NORS) stained with Ag represent ribosomal RNA synthesis which relates to synthetic activity of ribonucleoprotein. Derezini[31] explained that neoplastic cells were characterized by a large number of NORS which were small in size and showed a scattered distribution. Suarez[32] clarified that the number of nucleolus increased and the size grew larger with an average of 2.75 in gastric cancer cells in contrast with non-malignant cells of benign gastric ulcer in which the number of nucleolus was commonly one, sometimes two, rarely three and the size was 1.3 on the average.

In this study, the nucleolus was identified by light microscopy of a 100×2.5 to 100×5.0 manifying power without argyrophil staining. Few reports are now available for studies on the correlation between DNA pattern and the number of nucleolus in neoplastic cells. There was close relationship between increased nucleolus and aneuploid pattern. In this study, the survival rate within one year was better improved in the patients with the larger number of nucleolus than those in smaller number. A better survival rate was obtained in patients with diploid pattern which included the patients with the smaller number of nucleolus.

In conclusion, the worse prognosis following surgical treatment of Borrmann IV gastric cancer is based on aneuploid pattern on DNA histogram and the increasing number of nucleolus in neoplastic cells.

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