[総説] Cytometrical and morphologic heterogeneity in cell cycle-phased nuclei in a single glioma

Cytometrical and morphologic heterogeneity in cell cycle-phased nuclei in a single glioma

Yoshihiko Yoshii, Atsushi Saito, Hideo Tsurushima and Akio Hyodo

Department of Neurosurgery, Faculty of Medicine, University of the Ryukyus, Nishihara-cho, Okinawa 903-0215, Japan.

ABSTRACT

We examined nuclear morphometry and DNA content in SG2M phase tumor cells (including S phase and G2M phase cells) using image analysis to know whether or not cytometrical heterogeneity is always coupled with morphologic heterogeneity in 48 adult patients with glioma whose clinical follow-up was obtained. The SG2M phase cells were defined as the cells with a value (SD+0.1) above the mean diploid DNA indices. An estimated DNA index of the G2M phase cells (estimated G2M DNA index) was by multiplying the average G0G1 DNA indices by two. The G2M ploidy of each patient was classified into three patterns based on the difference between the mean SG2M DNA index and the estimated G2M DNA index: hypotetraploidy, tetraploidy or hypertetraploidy. Patients with G2M-hypertetraploid high grade gliomas had a significantly shorter time to tumor progression than patients with G2M-tetraploid tumors. The nuclear size of SG2M phase cells was larger and the nuclear shape was more markedly deformed than in G0G1 phase cells. However, there was no significant difference in the nuclear area or form factor in the SG2M nuclei between G2M-hypertetraploid and G2M-tetraploid cells with high grade glioma, high grade and low grade gliomas with G2M-tetraploid cell. Our results suggest that cytometrical heterogeneity does not always correlate with morphologic heterogeneity in glioma cells and that glioma cells with giant, multinucleated, or bizzare nuclei may have low proliferative potential. *Ryukyu Med. J., 19 (4)187~192, 2000*

Key words: human gliomas, G2M phase cell, densitometry, morphometry

INTRODUCTION

Despite improvements in diagnosis with the newly developed methods of neuroimaging, progress in the treatment of malignant gliomas remains at a standstill. Therapeutic failures in glioma treatment have largely been thought to result from biologically heterogenous responses to chemotherapy and radiotherapy in a single tumor[1].

Most of the evidence for heterogeneity in malignant brain tumors has thus far only been suggested by their pathologic appearance[2], however, there is data to support Nowell's hypothesis[3] that tumor progression and heterogeneity are the result of local mutations and clonal expansion arising from a monoclonal transformed astrocyte population[4]. Shapiro *et al* have demonstrated that karyotypic deviations were associated with morphologic heterogeneity of clonal sub-populations ranging from astrocyte-like to fibroblast-like[5]. It is important for prognosis, diagnosis, and treatment to know whether or not cytometrical heterogeneity is always coupled with morphologic heterogeneity in human gliomas. However, it is not known whether glioma cells with pleomorphic nuclei have an abnormal DNA content, or whether morphologic changes in the nuclei are related to the phase of the cell cycle.

Automated or semi-automated microscopic image analysis is an objective method that helps to detect differences which are hardly perceptible by other methods. Although these studies are limited to a small number of 100 cells[6,7], malignant cells can be preferentially selected for analysis. Furthermore, the image analysis allows for the simultaneous investigation of cytometric and morphometric information of the same tumor cell.

Our previous work with image analysis suggested that the relationship between SG2M phase tumor cells and clinical behavior was important, and that there are different stem cells in the aneuploid tumor cells of a brain tumor[8].

In the present study, we investigated histologic grade, SG2M phase fraction, G2M ploidy pattern, and stem cell lines in human gliomas. The time to tumor

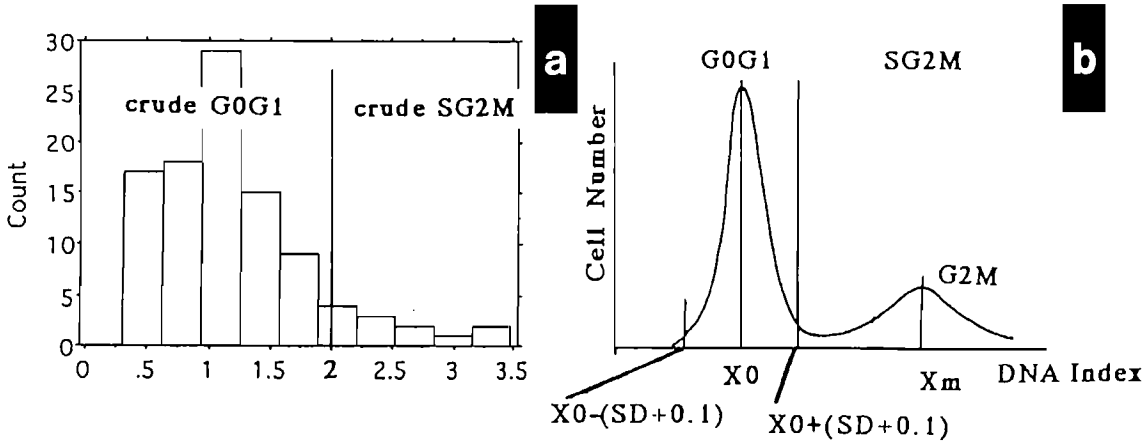

Fig. 1 Determination of G0G1 phase DNA index in a single glioma by TAS-plus.
The G0G1-phase cells was defined as a cell with nucleus of less than 2.0 DIs (a) and the first peak expressed the diploidy was determined as mean DNA indices of those cells and the diploid cells were considered to be cells with a nucleus in the range of mean ±(1SD+0.1)G0G1 DIs (b).

progression (TTP) of the patients was estimated as well. We describe here the relationship between the cytometrical heterogeneity and the morphologic heterogeneity of cell cycle-phased nuclei in a single glioma.

MATERIALS AND METHODS

Clinical Data and Histopathologic Diagnosis

All 48 adult patients included in the study underwent surgery at Tsukuba University Hospital. The histopathological classification of gliomas was determined by the Department of Pathology of our institution, according to the World Health Organization (WHO) classification. In this study, low grade glioma (LGG) indicated the WHO grade II astrocytoma, oligodendroglioma, and mixed glioma and/or the grade I or II gliomas according to Kernohan. Anaplastic glioma (AG) indicated the WHO grade III anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic mixed glioma and/or the grade III or IV gliomas according to Kernohan. Glioblastoma multiforme (GM) indicated the glioma with the features of anaplasia plus the presence of focal tumor necrosis and pseudopalisading. Twenty patients had glioblastoma multiforme, 17 patients had anaplastic gliomas, and 11 patients had low grade gliomas.

The patients with GM and AG were routinely treated with surgery and postoperative radiotherapy (RT) of 60-65 Gy combined with adjuvant chemotherapy. The patients with LGG also were treated routinely with surgery and postoperative RT. Time to tumor progression was estimated by the neurological deterioration and the appearance of the recurrence based on the neuroimages and/or the surgical specimen. The estimation of the clinical malignancy by time to tumor progression was correlated with DNA ploidy and proliferation index such as SG2 M fraction in all patients.

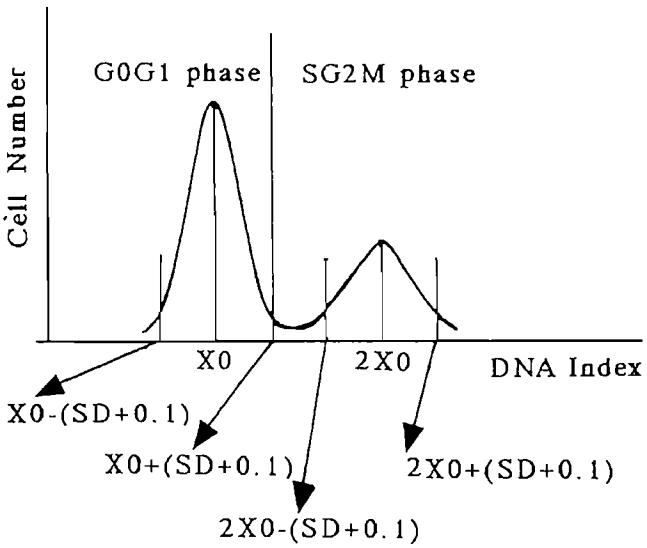

Fig. 2 Determination of G2M phase DNA indices in a single glioma by TAS-plus.
The estimated G2M DNA indices (2Xo) were obtained by multiplying the mean G0G1 DNA indices (Xo) by two. The range of tetraploid DNA indices was determined by the equation 2Xo± (SD+0.1).

Cytologic Samples and Cell Image Analysis

Samples obtained from the surgical resections were fixed in formalin and embedded in paraffin. Sections were cut serially from one block to 4μm thick. Some slides were processed routinely with PAS, Kluver-Barrera, reticulin silver impregnation, Azan-Mallory, Bodian, phosphotungstic acid hematoxylin (PTAH), H&E, or Feulgen staining. For Feulgen staining, the slide was brought to room temperature and rinsed in distilled water.

Cell image analysis was performed using a TAS plus microscope image processor (Leitz, Wetzlar, Germany) with a

Table 1 G2M ploidy, time to tumor progression, nuclear cytometry, and nuclear morphometry in 48 patients with glioma

G2M ploidy (number of patients)	Pathology	Time to tumor progression (month)	Nuclear cytometry			Nuclear morphometry in the SG2M nuclei	
			all DNA indices	SG2M-DNA indices	%SG2M	Area (μm^2)	Form factor
Hypotetraploidy (3)	Glioblastoma multiforme(1)	11	2.09	2.75	45	75.2	0.84
	Anaplastic glioma(2)	43±38.2	2.27±0.14	2.83±0.06	50±7.1	35.9±5.9	0.94±0.04
Tetraploidy (28)	Glioblastoma multiforme(9)	9.9±8.4[a,c]	1.82±0.51[d]	2.86±0.35[f,h]	32.2±16.8[i]	56.2±17.2	0.84±0.05
	Anaplastic glioma(8)	13.6±12.6[b]	1.94±0.71[e]	2.81±0.54[g]	35.9±23.1[j]	48.9±14.9	0.84±0.06
	Low Grade glioma(11)	45.6±21.5[a,b]	1.20±0.40[d,e]	2.27±0.39[f,g]	11.6±11.3[i,j]	47.7±11.0	0.87±0.06
Hypertetraploidy (17)	Glioblastoma multiforme(10)	4.2±4.0[c]	2.01±0.66	3.47±0.49[h]	34±16.8	60.3±17.3	0.80±0.08
	Anaplastic glioma(7)	10.6±5.4	2.16±1.27	3.53±0.93	37±29.7	61.3±26.9	0.80±0.06

a,f: $p<0.002$ b,h : $p<0.05$ c : $p=0.054$ d,i : $p<0.005$ e,g,j : $p<0.01$

x100 magnification lens (numerical aperature:1.32). The nuclear integrated optical density (IOD) was estimated on the densitometric levels on each pixel. The nuclear DNA content was calculated from the sum of the optical density (OD) values of each pixel of the nucleus. The IOD was measured using a wavelength of 545 nm. The nuclei of 100 well-preserved cells with neighboring nuclei were selected by visual observation and measured with image analysis. The IOD values of 10-20 lymphocytes from the same specimen were used to calculate the mean value of the control diploid cell population. That is, the DNA index (DI) of each tumor nuclei was determined by dividing the tumor-IOD by the mean IOD of the lymphocyte nuclei. The DI was expressed as arbitrary units. The morphometric nuclear parameters included form factor (FF), number of nuclei, and nuclear area (μm^2). FF was calculated as $4\pi \times$area/(perimeter) 2. An FF of 1.0 means that the nucleus is round, while an FF of 0.01 indicates an irregular shape. The nuclear size was estimated from the number of pixels occupied by a nucleus.

Determination of G2M Ploidy in Image Cytometry

Although various types of DNA distribution were observed in the FCM histograms, two modal such as diploidy and/or aneuploidy seemed to be important factor as a clinical prognosis. Cosidering the terminology adopted for the quantitative measurement of DNA, the term DNA aneuploid must be agreed on as being a synonym for abnormal stemline. Therefore, we think that it is essentially clinically important for tumor bilogy to be determined by DNA analyses whether the tumor has an abnormal stemline or not. Image cytometry (ICM) by TAS analysis dose not provide a standard G0G1 DI. Therefore, the G0G1 DIs were calculated by constructing a histogram of all DIs in the 100 measured nuclei. From this crude histogram we first determined the G0G1-phase cells with nuclei of less than 2.0 DIs (Fig. 1a). The diploid cells were considered to be those cells with a nucleus in the range of mean ± (1SD+0.1) G0G1 DIs (Fig. 1b). Then the SG2M phase cells (including S phase and G2M phase cells) were defined as the cells with a value (SD+0.1) above the mean diploid DNA indices. An estimated DNA index of the G2M phase cells (estimated G2M DNA index) is calculated by multiplying the average G0G1 DNA indices by two (Fig. 2). The G2M ploidy of each patient was classified into three patterns based on the difference between the mean SG2M DNA index and the estimated G2M DNA index: hypotetraploidy, tetraploidy or hypertetraploidy. For example, If those DIs were in a range (1SD+0.1) of estimated G2M DIs (2 times of G0G1 DIs), that peak would be equal to tetraploid G2M DIs. This means that that tumor had 2C and 4C amounts of DNA (Fig. 3a). If those DIs were above a range (1SD+0.1) of estimated G2M DIs, that peak would be equal to hypertetraploid G2M DIs. This means that that tumor has an abnormal stemline with nucleus more than 4C amount of DNA (Fig. 3c). If those DIs were below a range (1SD+0.1) of esimated G2M DIs, that peak would be equal to hypotetraploid G2M DIs (Fig. 3b). Thus, our method can estimate G2M and/or second DI peak from crude histogram of 100 tumor nuclei even though we had no standard G0G1 DI peak in ICM analysis.

A previous study of brain tumors[8] established a mean diploid DI of 1.2±0.32 in G0G1 phase tumor cells. We used this number to determine the ploidy pattern of the tumors in this series.

Statistical Analysis

Paired two-tailed Student's t-test was used for comparisons of the mean ±SD of the mean of the factors

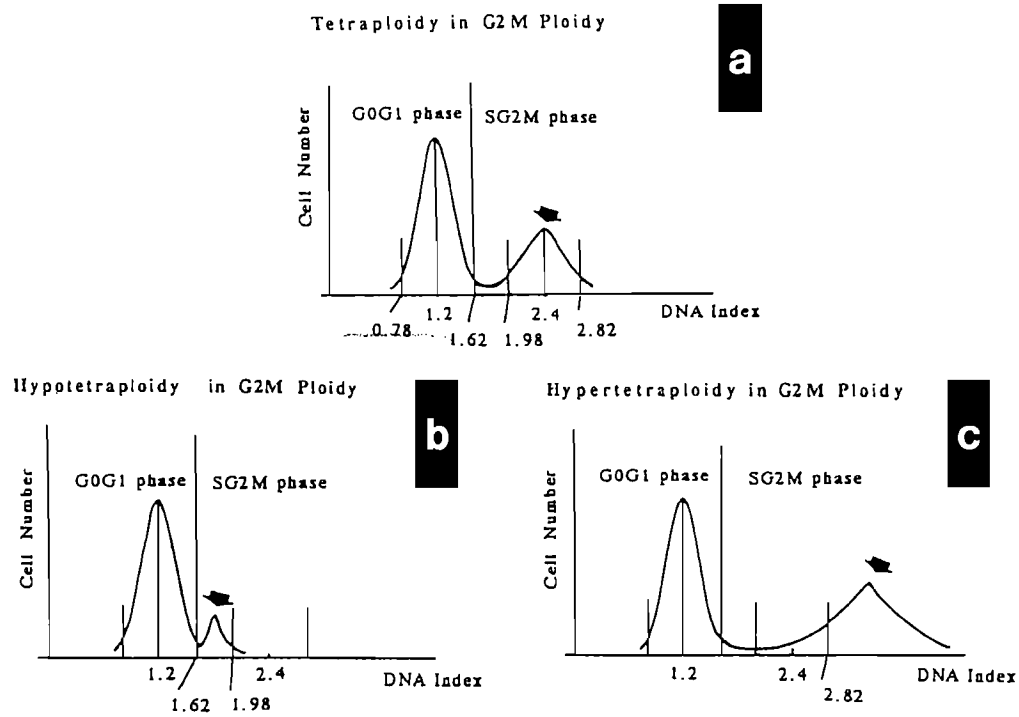

Fig. 3 Determination of ploidy represented by the mean DIs in all G2M phase cells.
a): Tetraploidy was defined as a calculated G2MDI within a range of ±(1SD +0.1) of the estimated G2MDI.
b): Hypotetraploidy indicates that the mean SG2MDI is less than 1SD + 0.1point below the estimated G2MDI.
c): Hypertetraploidy indicates that the mean SG2MDI is more than 1SD + 0.1point above the estimated G2MDI.

studied. Correlation and variation analysis were also used.

RESULTS

Morphometry in G0G1 and SG2M Phase Nuclei

In all the patients with gliomas studied, the mean area of nuclei in SG2M phase cells was significantly larger than in G0G1 phase cells (Fig. 4a). The mean FFs of nuclei in SG2M phase cells from patients with GM and AG were significantly lower than the mean FF of G0G1 phase cells. A similar pattern was seen in LGG, but the difference was not significant (Fig. 4b).

Nuclear Cytometry and Morphometry in SG2M Phase Cells

In patients with tetraploid gliomas, the patients with high grade glioma had a shorter mean TTP, a higher mean DI, a higher mean SG2MDI, and a higher mean %SG2M than those with low grade glioma ($p<0.05$-0.002) (Table 1).

The patients with GM and hypertetraploid tumors had a shorter mean TTP and a higher mean SG2MDI than those with tetraploid tumor cells ($p=0.054$, $p<0.05$).

In the 37 patients with high grade glioma, the patients with hypertetraploid tumors had a shorter mean TTP than those with tetraploidy tumors ($p<0.05$) (Table 2). There was no significant difference in TTP between patients with hypotetraploidy and tetraploidy, or between patients with hypertetraploidy and hypotetraploidy, although the three patients with hypotetraploid tumors had a tendency for a longer TTP. The mean SG2MDIs of patients with hypertetraploid gliomas were significantly higher than those of patients with tetraploid tumors. However, the %SG2M values were not significantly different between the patients with tetraploid and hypertetraploid gliomas.

In the patients with tetraploid gliomas, the patients with high grade gliomas had a shorter mean TTP and a higher mean %SG2M than those with low grade gliomas (Table 1). However, there was no significant difference in the nuclear area or form factor in the SG2M nuclei between G2M-hypertetraploid and G2M-tetraploid cells with high grade glioma, high grade and low grade gliomas with G2M-tetraploid cell (Table 1,2).

DISCUSSION

The degree of nuclear atypism in the tumor cells may be correlated with the degree of malignancy in

Table 2 G2M ploidy, time to tumor progression, nuclear cytometry, and nuclear morphometry in 37 patients with high grade glioma

G2M ploidy (number of patients)	Time to tumor progression (month)	Nuclear cytometry			Nuclear morphometry in the SG2M nuclei	
		all DNA indices	SG2M-DNA-indices	%SG2M	Area(μm^2)	Form factor
Hypotetraploidy (3)	32.3±32.7	2.21±0.14	2.8±0.06	48.3±5.8	48.4±23.6	0.90±0.06
Tetraploidy (17)	11.7±10.4[a]	1.88±0.59	2.84±0.43[b]	33.9±19.4	52.8±16.1	0.84±0.05
Hypertetraploidy (17)	6.8±5.5[a]	2.07±0.93	3.49±0.68[b]	35.2±22.1	60.7±21.0	0.80±0.07

a : $p<0.05$ b : $p<0.01$

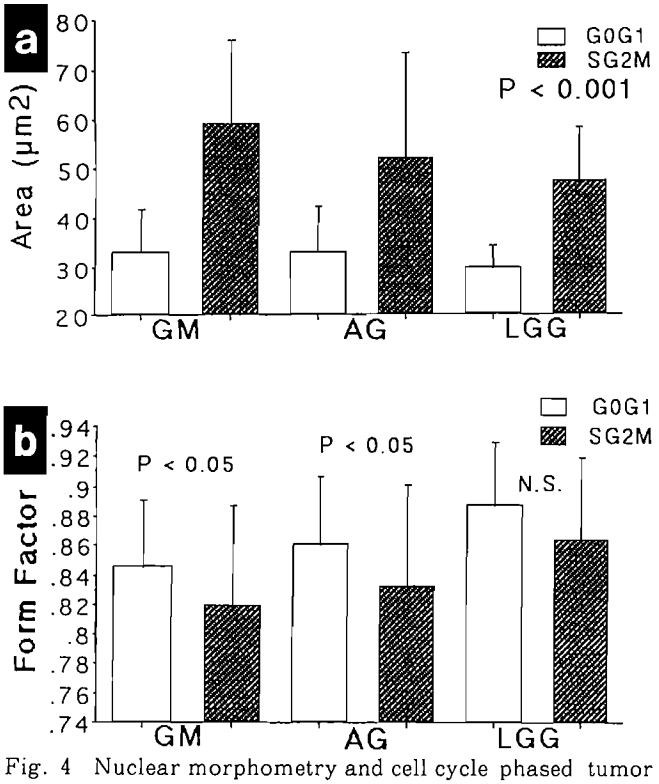

Fig. 4 Nuclear morphometry and cell cycle phased tumor cell.

a): Nuclear area in SG2M phase cells was significantly larger than in G0G1 phase cells in all gliomas studied ($p< 0.001$).

b): Form factors of nuclei in SG2M phase cells from patients with glioblastoma multiforme and anaplastic glioma were significantly lower than that of G0G1 phase cells ($p< 0.05$).

GM: glioblastoma multiforme, AG: anaplastic glioma, LGG: low grade glioma, G0G1: G0G1 phase cell, SG2M: S phase and G2M phase cell.

gliomas. Martin *et al* have reported that increasing nuclear size is seen with increasing degrees of malignancy in gliomas, and that a large variability in nuclear volume may be a quantitative expression of aneuploidy[9]. On the other hand, Levi *et al* and Peitzer *et al* have reported that nuclear size is not a reliable marker of polyploidy[10, 11].

However, we found that the nuclear area was larger and the form factor showed more marked deformity in SG2M phase cells than in G0G1 phase cells in GM and AG. This may result from the high cellular metabolism of SG2M phase cells, and may not be a direct consequence of the number of chromosomes, as stressed by Levi *et al* and Pfitzer *et al*[10,11]. In support of this idea, nuclear morphology in our study was not significantly different between hypertetraploid and tetraploid tumor cells. Hypertetraploid glioma cells therefore had an abnormal DNA content without any variability in nuclear morphology. Thus, our findings are in agreement with those of Levi *et al* and Pfitzer *et al*[10,11].

However, our data also indicated that there was no significant difference among the nuclear sizes of tumor cells with more than 2C DNA content. These results suggest that glioma cells with giant, multinucleated, and bizzare nuclei have low proliferative potential. Therefore, we agree that the appearance of pleomorphic nuclei in glioma tissue may not be a more reliable prognositic factor than necrosis or the number of mitoses[12, 13]. Nuclear volume may be regulated by mechanisms other than DNA content[14]. The analysis of heterogeneity in glioma cells appears to have contributed to improvements in the therapy of these tumors. However, cytometrical heterogeneity does not always correlate with morphologic heterogeneity in glioma cells. Our studies suggest that morphologic heterogeneity in gliomas needs to be further investigated in relation to other processes, such as apoptosis.

When the DNA distribution of tumor cells are analyzed using a flow cytometry (FCM), cells containing a larger amount of DNA content than normal diploid cells are observed. All tumors with more than 20% cells in the hypodiploid and/or hyperdiploid zones, or with bimodal, multimodal, and amodal distribution in the histograms were considered aneuploid.

The investigations have been classified into the different mode with DNA ploidy, for example diploid, near diploid, aneuploid and so on. However, the subdivision of DNA ploidy was not reliable to determine biological

features of tumor cells such as stem lines. For the cytometric studies, it is clinically importance to determine whether the tumor has an abnormal stem line or not. Our method can estimate G2M and/or second DI peak from crude histogram of 100 tumor nuclei even though we had no standard G0G1 DI peak in ICM analysis.

REFERENCES

1) Bigner D.D.: Biology of gliomas: Potential clinical implications of glioma cellular heterogeneity. Neurosurgery. 9: 320-326, 1981.
2) Burger P.C. and Vollmer R.T.:Histologic factors of prognostic significance in the glioblastoma multiforme. Cancer. 46: 1179-1186, 1980.
3) Nowell P.: The clonal evolution of tumor cell populations. Science. 194: 23-28, 1976.
4) Berkman R.A., Clark W.C., Saxena A., Robertson J.T., Oldfield E.H. and Ali I.U.: Clonal composition of glioblastoma multiforme. J. Neurosurg. 77: 432-437, 1992.
5) Shapiro J.R., Yung W.K.A. and Shapiro W.R.: Isolation, karyotype, and clonal growth of heterogenous subpopulations of human malignant gliomas. Cancer Res. 41: 2349-2359, 1981.
6) Cope C., Rowe D., Delbridge L., Philips J. and Friedlander M.: Comparison of image analysis and flow cytometric determination of cellular DNA content. J. Clin. Pathol., 44: 147-151, 1991.
7) Mikel U.V. and Becker R.L.: A comparative study of quanititative stains for DNA in image cytometry. Anal. Quan. Cytol. Histol. 13: 253-259, 1991.
8) Yoshii Y., Saito A., Tsuboi K., Tsurushima H., Komatsu Y., Tomono Y. and Nose T.: Nuclear characterization and G2M ploidy in human brain tumors using semiautomated image analysis. Brain. Tumor. Pathol.12:61-67, 1995.
9) Martin H., Voss K., Hufnagl P. and Frolich K.: Automated image analysis of gliomas an objective and reproducible method for tumor granding. Acta Neuropathol. (Berl) 63: 160-169, 1984.
10) Levi P.E., Cooper E.H., Anderson C.K., Path M.C. and Williams R.E.: Analyses of DNA content, nuclear size, and cell population of transitional cell carcinoma in man. Cancer 23: 1074-1085, 1969.
11) Pfitzer P. and Page H.D.: Characterization of tumor cell populations by DNA-measurements. Acta. Cytol. 17: 19-26, 1973.
12) Burger P.C., Vogel S., Green S. and Strike T.: Glioblastoma multiforme and anaplastic astrocytoma. Pathological criteria and prognostic implications. Cancer 56: 1106-1111, 1985.
13) Daumas- Duport C., Scheithauer B., O'Fallon J. and Kelly P.: Grading of astrocytomas: a simple and reproducibe method. Cancer 62: 2152-2165, 1988.
14) Miles C.P. and Koss L.G.: Diagnostic traits of interphase human cancer cells with known chromosome patterns. Acta Cytol. 10: 21-25, 1966.