Yonsei Medical Journal
Vol. 35, No. 1, 1994

Flow Cytometric Analysis of DNA Ploidy in Childhood Rhabdomyosarcoma

Woo-Hee Jung, Soon Hee Jung[1], Cheol Joo Yoo[2], Yee Jeong Kim Chanil Park and Byung Soo Kim[2]

Flow cytometric DNA analysis was performed on 17 rhabdomyosarcomas in conjunction with a histopathological review to determine the usefulness of this technique to predict the biologic behavior of the tumor and to establish the characteristic ploidy pattern of rhabdomyosarcoma compared to other small round cell tumors occurring in childhood. Aneuploidy including near-tetraploidy is the most common ploidy pattern encountered, followed by multiploidy and diploidy, and the presence of multiploidy in this tumor is useful for differentiating rhabdomyosarcoma from other kinds of small round cell tumors in which there are rare previous reports on occurrence of multiploidy. Even though there is no significant correlation between ploidy pattern and histologic type of rhabdomyosarcoma, patients with multiploid tumors or aneuploid tumors with a DNA index of 1.10-1.80 tend to have a high risk of treatment failure. Therefore, the ploidy pattern seems to be useful for predicting the patient's survival in concert with other variables.

Key Words: Rhabdomyosarcoma, flow cytometry, DNA content, histopathology

Rhabdomyosarcoma is one of the most common soft tissue sarcoma in children, accounting for 4% to 8% of all childhood malignancies in patients under 15 years of age (Young and Miller, 1975). It is well known that prognosis of rhadomyosarcoma has improved dramatically over the past two decades due to the introduction of combined modality therapy, consisting of the surgical removal of the tumor, radiation therapy, and multiagent chemotherapy.

Currently, a few flow cytometric studies have been performed to prove that DNA ploidy of rhabdomyosarcoma may be used as an additional prognostic indicator in conjunction with the primary site, histologic type, and clinical stage (extent of disease) (Boyle *et al.* 1988; Molenaar *et al.* 1988; Kowal-Vern *et al.* 1990; Shapiro *et al.* 1991). However, there are conflicting reports on the relationship of ploidy pattern to the patient's survival: one report (Kowal-Vern *et al.* 1990) suggests that patients with an aneuploid tumor have poor survival by contrast with another report (Shapiro *et al.* 1991) suggesting that patients with an aneuploid tumor with a DNA index of 1.10-1.80 have better survival. Furthermore, the number of patients studied previously were small and the results were not that consistent so as to be used as a therapeutic guideline in the treatment of patients with rhabdomyosarcoma.

In our study, flow cytometric DNA analysis was performed retrospectively on 17 cases of rhabdomyosarcoma. We wished to establish the characteristic ploidy pattern of rhabdomyosarcoma in comparison with that of

Received September 23, 1993

Departments of Pathology and Pediatrics[2], Yonsei University College of Medicine, Seoul, Korea

[1]Department of Pathology, Yonsei University Wonju College of Medicine, Wonju, Korea

This study was supported by Institute for Cancer Research Grant(1992), Yonsei Cancer Cetner.

Address reprint requests to W-H Jung, M.D., Department of Pathology, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul, Korea, 120-752

other small round cell tumors reported previously and to establish the relationship of ploidy pattern (DNA index) to histologic type and the patient's survival.

MATERIALS AND METHODS

Rhabdomyosarcomas from 30 patients under 15 years of age diagnosed between 1982 and 1991 were retrieved from the files of the Department of Pathology, Yonsei University College of Medicine, Seoul, Korea. Only 17 of 30 cases were available for flow cytometric DNA analysis with optimal histograms. The medical records, gross descriptions, and microscopic slides stained with hematoxylin-eosin and with immunohistochemical stain for desmin were reviewed. All patients were registered and followed up at Yonsei Cancer Center, reflecting as much standardization of therapy as possible. Each patient was treated by the Intergroup Rhabdomyosarcoma Study (IRS) protocol, consisting of postoperative chemotherapy with vincristine, dactinomycin and cyclophosphamide(VAC) and radiotherapy if needed (Pizzo and Poplack, 1989).

Histologic and immunohistochemical evaluation

In four of 17 cases, specimens were obtained by biopsy and only one or two paraffin blocks were available and the remaining 13 were obtained by partial or complete excision and 5 to 26 blocks were available for examination. All specimens were fixed in 10% neutral formalin, embedded in paraffin, cut with 4 to 5 micron sections and stained with hematoxylin-eosin. All tumors were classified into embryonal, alveolar, anaplastic and mixed types according to growth pattern and cytologic characteristics proposed by Jung *et al* (1992). Immunohistochemistry for desmin was performed to facilitate pathologic diagnosis using the labelled streptavidine biotin kit (LSAB, Dako).

Flow cytometric DNA analysis

Formalin-fixed, paraffin-embedded samples of tumor were selected for flow cytometric DNA analysis. Flow cytometric DNA analysis (DNA FCM) was performed on isolated nuclei using a modification of the method of Headley *et al* (1985). Two or three 50 micron sections cut from the tissue blocks were deparaffinized in Histo-Clear (National Diagnostics, Manville, NJ) and rehydrated in a series of graded alchohols. Disaggregation of nuclei was accomplished by treatment with 2.5 ml of 0.5% pepsin (Sigma Chemical Corp., St. Louis, MO) at PH 1.5 at 37℃ for 30 minutes with intermittent vortexing. Digestion was stopped by adding 1 ml of a 0.005% peptostatin solution (Sigma Chemical Corp. St. Louis, MO). The samples were then centrifuged at 2,000 rpm, washed twice with Dulbecco's phosphate buffered saline (Sigma Chemical Corp., St. Louis, MO), and incubated with freshly prepared 0.5 ml of RNAse (2.50 mg/ml; Worthington Biochemical, Freehold, NJ) at 37℃ for 30 minutes. The samples were filtered through a 50 micron nylon mesh filter (Small Parts Inc., Miami, FL), and stained with 0.025% propidium iodide (Sigma Chemical Corp. St. Louis, MO) at 50 ul/Ml. The isolated nuclei were adjusted to a concentration of 1 to 3×10^6/ml by diluting with Dulbecco's phosphate buffered saline.

Nuclei were analyzed in a FACScan (Beckton-Dickinson, Sunnyvale, CA) with at least 10,000 nuclei read per sample. The first G0/G1 peak was assumed to be the diploid population and assigned a DNA index of 1.0. DNA aneuploidy was defined by the presence of a distinct, separate second peak to the right of the first G0/G1 peak followed by a low G2M peak in the hexaploid to octaploid range. The half-peak coefficient of variance (CV) ranged from 3.4% to 5.6%. The DNA index was calculated by the ratio of the channel number of the abnormal aneuploid peak to that of the normal diploid G_0/G_1 peak. The term 'multiploidy' was used to refer to the presence of two or more aneuploid peaks in one tumor. In a multiploid tumor, the DNA index of each aneuploid peak can be obtained in the same manner. 'Near-tetraploidy' denoted a tumor stem line with a DNA index between 1.80 and 2.20.

Statistical analysis

Fisher's exact test, Kaplan-Meier analysis for survival curve and the Cox proportional

hazard model for multivariate analysis were used for statistical evaluation. A difference was regarded as statistically significant if p was less than 0.05.

RESULTS

The clinical, histopathologic and flow cytometric data of each patient are summarized in Table 1. The cases are arranged by grouping of ploidy pattern i. e., multiploidy, aneuploidy, near-tetraploidy and diploidy.

Clinical Feature

Age and Sex of Patients
Range: 4 months to 15 years (median: 7 years)
10 males and 7 females

Number of Tumors by Primary Site
Head and Neck : 4
Intraabdominal and Pelvic : 4
Trunk : 3
Testis : 1
Extremities : 5

Number of Tumors by Stage
Stage I : 2
Stage II : 5
Stage III : 6
Stage IV : 2
Unknown : 2

Follow-up and mortality

Four patients failed in correspondence in their follow-up period, nine patients died of their disease in the course of the follow-up periods from 4 months to one year and 8 months and four patients either survived more than 5 years or had no evidence of dis-

Table 1. Study patient characteristics

No.	Age(yr)/sex	Site	Stage	Histologic type	Ploidy	DNAI	Follow-up period	Status
1	4/12/M	Face	III	E	M	1.87, 3.54	7 months	DOD
2	2.11/12/M	Thigh	II	M	M	1.52, 2.04	1 8/12 yrs	DOD
3	3.10/12/F	Pelvic mass	III	Ap	M	1.91, 2.79, 5.32	12 months	DOD
4	4.7/12/M	Cheek	III	A	M	1.43, 2.54	12 months	DOD
5	5.7/12/M	Retro-peritoneum	III	E	M	1.20, 1.92	2 2/12 yrs	NED
6	4/M	Thorax (intercostal muscle)	II	Ap	An	1.73	5 months	DOD
7	13.6/12/F	Nasopharynx	III	E	An	1.39	7 11/12 yrs	NED
8	14/M	Testis	?	E	An	1.22		NA
9	14.8/12/F	Perianal	I	A	An	1.21	4 months	DOD
10	7/F	Paravertebral	?	Ap	An	1.20		NA
11	15/F	Abdominal & chest wall	IV	E	An	1.73	6 months	DOD
12	3.3/12/F	Popliteal fossa	I	Ap	T	2.14	2 2/12 yrs	NED
13	3.3/12/M	Parotid	III	E	T	1.84	7 6/12 yrs	NED
14	3.8/12/M	Calf	II	M	T	1.84		NA
15	14.3/12/F	Axilla, back	II	A	T	1.98	1 4/12 yrs	DOD
16	10/M	Axilla, arm	IV	A	D	1.0	5 months	DOD
17	12/M	Thigh	III	E	D	1.0		NA

E: Embryonal A: Alveolar Ap: Anaplastic M: Mixed
D: Diploidy An: Aneuploidy T: Near-tetraploidy
M: Multiploidy DOD: Died of disease DNAI: DNA index
NED: No evidence of disease more than 2 years after finishing treatment
NA: Not available

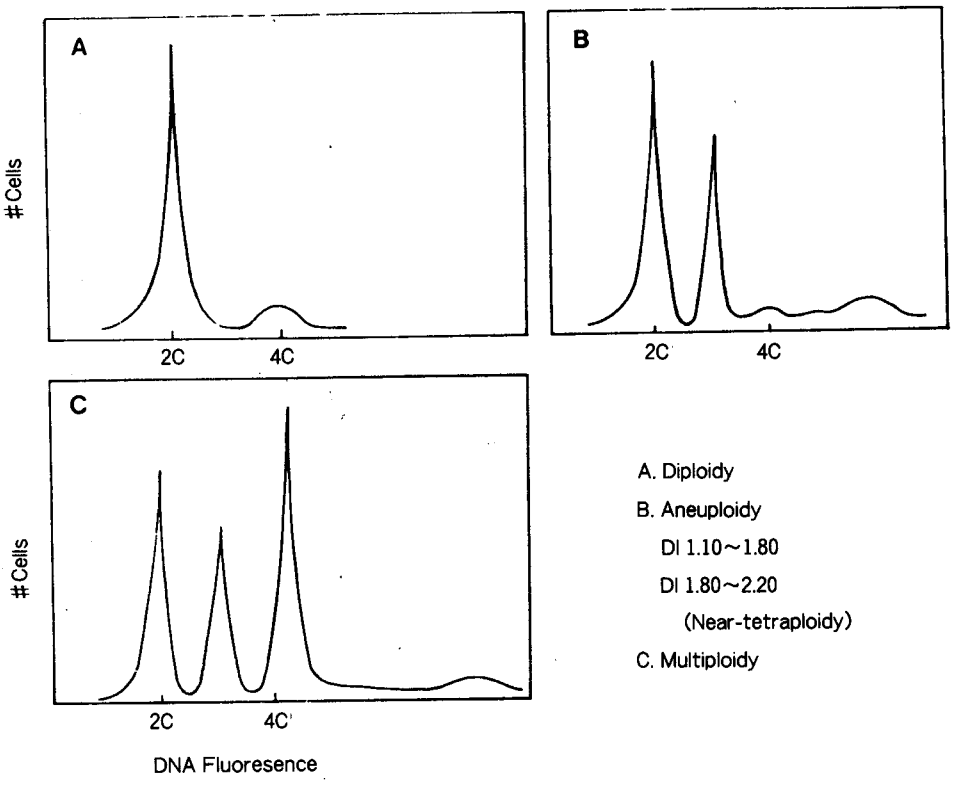

Fig. 1. *Nuclear DNA histogram patterns of rhabdomyosarcoma.*

ease more than 2 years after finishing the treatment.

Histologic and immunohistochemical feature

Histologic types were embryonal in 7(40%), alveolar in 4(24%) and mixed in 2(12%). Cellular anaplasia was noted in 4(24%) cases with embryonal histology. Tumor cells were positive for desmin with a scattered or diffuse pattern in all cases, facilitating the confirmation of the diagnosis of rhabdomyosarcoma.

Ploidy pattern of rhabdomyosarcoma

The nuclear DNA histogram patterns we have encountered in 17 rhabdomyosarcomas and the distribution of the ploidy pattern are depicted in Fig. 1 and Fig. 2. Of 17 tumors, only two (12%) were diploidy with a DNA index of 1.0. Ten (59%) tumors showed a distinct DNA aneuploid peak with a DNA index ranging from 1.20 to 2.14; six had a DNA index ranging from 1.10 to 1.80 and four from 1.80 to 2.20. Five (29%) tumors showed two or more aneuploid peaks (i.e. mul-

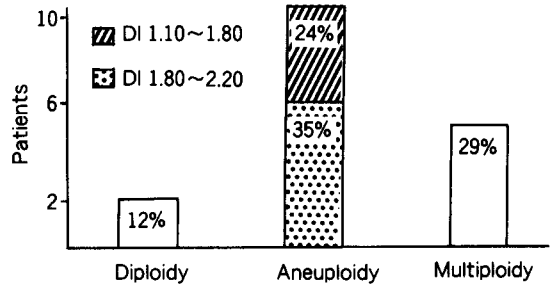

Fig. 2. *Distribution of ploidy pattern in rhabdomyosarcoma.*

Table 2. DNA ploidy related to histologic types

	Embryonal	Alveolar	Anaplastic	Mixed	
Diploidy	1	1	0	0	2(12)
Aneuploidy	4	2	3	1	10(59)
DI 1.10~1.80	3	1	2	0	6(35)
DI 1.80~2.20	1	1	1	1	4(24)
Multiploidy	2	1	1	1	5(29)
Total	7(40)	4(24)	4(24)	2(12)	17(100)

( )=percentage

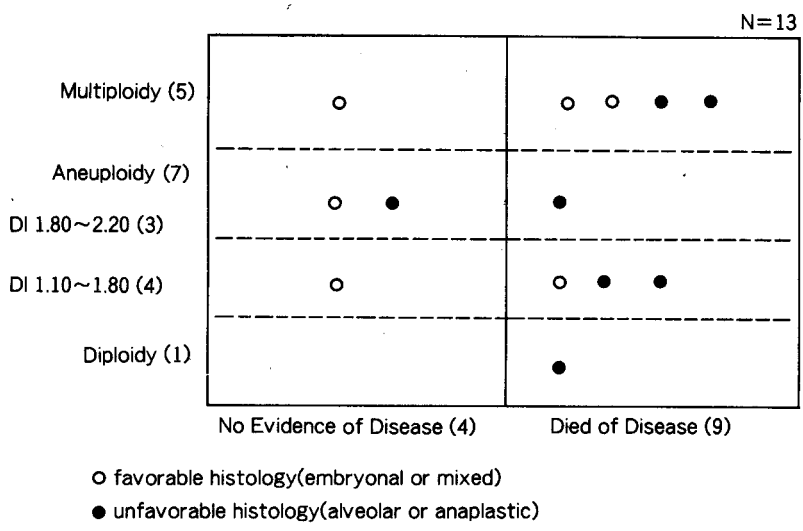

***Fig. 3.** Survival related to DNA ploidy.*

tiploidy). In our rhabdomyosarcomas, aneuploidy is most commonly found, followed by multiploidy and diploidy and for the aneuploid tumors, 40% have a near-tetraploid or tetraploid DNA index.

DNA ploidy related to histologic type

Four of seven tumors with embryonal histology showed aneuploidy including one near-tetraploidy, two multiploidy, and one diploidy. Four tumors with alveolar histology showed one diploidy, two aneuploidy including one near-tetraploidy and one multiploidy respectively. Of four tumors with an anaplastic variant of embryonal histology, three showed aneuploidy including one near-tetraploidy and one multiploidy. Two tumors with mixed histology had near-tetraploidy and multiploidy each. There is no significant correlation between DNA ploidy and histologic type(Table 2).

Patient's survival related to DNA ploidy

Thirteen of seventeen patients were available for clinical follow-up. Four patients either survived more than five years or had no evidence of disease more than two years after finishing the treatment, and nine patients died of their disease. Four of five patients with a multiploid tumor and three of

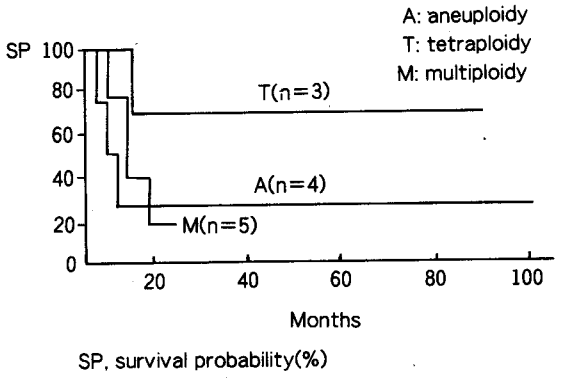

Fig. 4. *Kaplan-Meier analysis of overall survival by ploidy pattern for 12 patients.*

four patients with an aneuploid tumor with a DNA index ranging from 1.10 to 1.80 died of their disease. By contrast, one of three patients with a near-tetraploid tumor died of the disease. The difference in survival between the two groups is not statistically significant($p=0.236$). Six of seven patients with tumors with unfavorable histology such as the alveolar or anaplastic type died of their disease; in contrast three of six patients with tumors with favorable histology such as the embryonal or mixed type died of their disease. The difference in survival between the two groups is not statistically significant($p=0.266$). Patients with a multiploid tumor or aneuploid tumor with a DNA index of 1.10~1.80 tended to have a high risk of treatment failure, although not statistically significant by multivariate analysis (Fig. 3, Fig. 4).

DISCUSSION

Rhabdomyosarcoma in childhood used to have a very poor prognosis in that extensive local invasion and early distant metastasis are very frequent in this tumor, which dramatically improved after the introduction of multimodality treatment.

It has been well known that aneuploidy in human tumors, including sarcomas, often correlates with poor survival as compared to otherwise diploid tumors. In contrast with adult tumors, aneuploid tumors appear to have a better prognosis in pediatric tumors such as neuroblastoma, acute lymphoblastic leukemia and medulloblastoma (Look *et al.* 1984; Look *et al.* 1985; Tomita *et al.* 1988). However, in the current studies on DNA ploidy in a group of rhabdomyosarcomas including different histological subtypes, there are conflicting reports on the relationship of ploidy pattern to the patient's survival. One report (Kowal-Vern *et al.* 1990) suggests that DNA aneuploidy is not a predictor of a good prognosis by contrast with another report (Shapiro *et al.* 1991) suggesting that patients with aneuploid tumor with a DNA index of 1.10~1.80 have better survival; 75% of patients with hyperdiploid rhabdomyosarcomas are long-term survivors, whereas no patients with diploid tumors survived for longer than 18 months. In one study (Boyle *et al.* 1988), 13 patients with embryonal rhabdomyosarcomas of the bladder and prostate showed all aneuploid stem lines of tumor cells with a DNA index ranging from 1.21 to 2.49 and six of which treated since 1971 receiving preoperative chemotherapy (vincristine, doxorubicin and cyclophosphamide; VAC) have remained well with a mean survival of 73.8 months. Conversely, 6 of 7 patients treated before the era of chemotherapy with surgery alone had a rapid tumor recurrence and died of disease of a median of 5.5 months post-treatment regardless of the DNA index. This confirms the enhanced survival demonstrated in patients undergoing primary chemotherapy previously by the Intergroup Rhabdomyosarcoma Studies (Kramer, 1985). Furthermore, Molenaar *et al* (1988) concluded that DNA aneuploidy is related on the one hand to the generally aggressive clinical behaviour and on the other hand to the favorable response to multimodality treatment. In our small study, patients with a multiploid tumor or aneuploid tumor with a DNA index of 1.10~1.80 tended to have a high risk of treatment failure, although not statistically significant. Though the present series, including ours, is apparently too limited to draw firm conclusions, it does not seem likely that flow cytometric ploidy analysis will be useful in this particular tumor type for categorizing patients into prognostic groups without considering the other prognostic variables.

Table 3. DNA ploidy in rhabdomosarcoma

Authors	Total no. of Patients	No. of		% Aneuploidy
		Diploidy	Aneuploidy	
Present study	17	2	15(4/5)	88
Boyle(1988)	*13*	*0*	*13(0/0)*	*100*
Molenaar(1988)	11	0	11(2/3)	100
Kowal-Vern(1990)	20	4	12(*4/4)	60
Shapiro(1991)	37	11	26(11/10)	70
Total	98	17	77	79

Parenthesis incidates no. of near-tetraploidy/multiploidy
*Polyploidy

The finding of a high frequency of aneuploidy including near-tetraploidy and multiploidy in rhabdomyosarcomas demonstrated in our selected study population, is in keeping with other studies with similar constraints (Tabel 3). When one considers previously reported cases of rhabdomyosarcomas as well as those studied by us, overall, aneuploidy is found commonly in 79%. The presence of multiploidy in rhabdomyosarcoma also was demonstrated in two other reports (Molenaar *et al.* 1988; Kowal-Vern *et al.* 1990); two or more different aneuploid peaks were observed in 3 of 11 (27%) and 4 of 20 (20%) patients respectively, which appears similar to ours (29%). Frequent or rare occurrences of multiploidy is reported in various kinds of adulthood malignancies of breast, ovary, endometrium and soft tissue (Bauer *et al.* 1993). However, there is no report of the presence of multiploidy with considerable frequency in pediatric small round (blue) cell tumors except for rhabdomyosarcoma. Furthermore, Ewing's sarcoma and primitive neuroectodermal tumor (PNET) appear to have diploid or near-diploid stemlines sharing at (11:22) translocation by cytogenetic study (Turc-Carel *et al.* 1984; Whang-Peng *et al*, 1984). This suggests that the DNA ploidy may be of additional help in arriving at a correct differential diagnosis between poorly differentiated rhabdomyosarcoma and other primitive childhood tumors, such as Ewing's sarcoma and PNET, when differential diagnosis is especially difficult histologically.

In our small study, there is no significant correlation between histologic subtypes related to prognosis and ploidy pattern. However, interestingly all four patients with the anaplastic variant of embryonal rhabdomyosarcoma showed a definite aneuploid peak including one multiploidy. Consistent karyotypic findings have been reported in cytogenetic studies of rhabdomyosarcoma, which revealed a frequent occurrence of hyperdiploidy with near-tetraploid clones predominating and a specific chromosomal abnormality, t(2; 13) (q 35; q 14), which had not been identified in other pediatric solid tumors investigated so far (Douglass *et al.* 1987; Sheng *et al.* 1988). The only specific chromosomal translocation that has been observed in a pediatric solid tumor is the t(11:12), found in both Ewing's sarcoma and PNET (Turc-Carel *et al.* 1984; Whang-Peng *et al.* 1984). The flow cytometric DNA content of the tumor cannot be directly translated into number of chromosomes or other karyotypic features such as translocation, double minute bodies and homogeneously staining regions, presumably indicating gene amplification. However, in one study (Shapiro *et al.* 1991) stem-line DNA indices were proven to be correlated with modal numbers of chromosome by cytogenetic analysis of rhabdomyosarcoma. In addtion, they demonstrated that hyperdiploidy was a characteristic feature of embryonal rhabdomyosarcoma and by contrast, the alveolar subtype was associated almost exclusively with near-tetraploid DNA content in agreement with a flow cytometric study of 11 patients with rhabdomyosarcoma by Molenaar *et al.*

(1988), who disclosed that all five of the alveolar tumors they examined contained near-tetraploid stem lines, whereas five of the six embryonal tumors were hyperdiploid.

The latest report by Shapiro *et al.* (1991), who performed combined flow cytometric and cytogenetic studies suggests that there are two major classes of rhabdomyosarcoma distinguished by histologic subtype, DNA ploidy, cytogenetic abnormalities and response to chemotherapy and prognosis. Rhabdomyosarcoma with embryonal type, DNA aneuploidy (intermediate hyperdiploidy), absence of translocation (2:13) or gene amplification and a good response to treatment protocols are characteristics of the favorable prognosis group. Contrasted with this are tumors with the alveolar type, DNA diploidy or near-tetraploidy, presence of translocation (2:13) or gene amplification and high risk for treatment failure.

In conclusion, the ploidy pattern, which is characterized by the high frequency of aneuploidy and the frequent presence of multiploidy, is useful for differentiating this tumor from other kinds of small round cell tumors in childhood and there is no significant correlation between the ploidy pattern and histologic subtype. Although involving only a limited series, these results suggest that the anlaysis of DNA ploidy in concert with other variables could help in predicting the clinical outcome in rhabdomyosarcoma and identifying patients for whom a more aggressive therapy is required. We hope that these flow cytometric findings including ours could suggest directions for further investigation of molecular events underlying the genesis of rhabdomyosarcoma.

REFERENCES

Bauer KD, Duque RE, Shankey TV: *Clinical flow cytometry.* Principles and application. Williams and Wilkins, Baltimore.

Boyle ET, Reiman HM, Kramer SA, Kelalis PP, Rainwater LM, Loeber MM: Embryonal rhabdomyosarcoma of bladder and prostate: nuclear DNA patterns studied by flow cytometry. *J Urol 140: 1119-1121, 1988*

Douglass EC, Valentine M, Etcubanas E, Parham D, Webber BL, Houghton PJ, Green AA: A specific chromosomal abnormality in rhabdomyosarcoma. *Cytogenet Cell Genet 45: 148-155, 1987*

Headley DW, Friedlander ML, Taylor IW: Application of DNA flow cytometry to paraffin-embedded archival material for the study of aneuploidy and its clinical significance. *Cytometry 6: 327-333, 1985*

Jung WH, Kim YJ, Jung SH, Yim H, Yoo CJ: Rhabdomyosarcoma in children; histologic subtypes and prognosis. *Korean J Pathol 26; 573-581, 1992*

Kowal-Vern A, Gonzalez-Crussi F, Turner J, Trujillo YP, Chou P, Herman C, Castelli M, Walloch J: Flow and image cytometric DNA analysis in rhabdomyosarcoma. *Cancer Res 50: 6023-6027, 1990*

Kramer SA: Pediatric urologic oncology. *Urol Clin N Amer 12: 31-42, 1985*

Look AT, Hayes FA, Nitschke R, McWilliams NB, Green AA: Cellular DNA content as a predictor of response to chemotherapy in infants with unresectable neuroblastoma. *N Engl J Med 311: 231-235, 1984*

Look AT, Roberson PK, Williams DL, Rivera G, Bowman WP, Pui C-H, Ochs J, Abromowitch M, Kalwinski D, Dahl GV, George S, Murphy SB: Prognostic importance of blast cell DNA content in childhood acute lymphoblastic leukemia. *Blood 65: 1079-1086, 1985*

Molenaar WM, Dam-Meiring A, Kamps WA, Cornelisse CJ: DNA aneuploidy in rhabdomyosarcomas as compared with other sarcomas of childhood and adolescence. *Hum Pathol 19: 573-579, 1988*

Newton WA, Soule EH, Hamoudi AB, Reiman HM, Shimada H, Beltangady M, Mauer H: Histopathology of childhood sarcomas, Intergroup Rhabdomyosarcoma Studies I & II: clinicopathologic correlation. *J Clin Oncol 6: 67-75, 1988*

Pizzo PA, Poplack DG: *Principles and practice of pediatric oncology.* Philadelphia, JB Lippincott Co, *1989, small PP 635-658.*

Shapiro DN, Parham DM, Douglass EC, Ashumn R, Webber BL, Newton WA, Hancock ML, Maurer HM, Look AT: Relationship of tumor cell ploidy to histologic subtype and treatment outcome in children and adolescents with unresectable rhabdomyosarcoma. *J Clin Oncol 9: 159-166, 1991*

Sheng WW, Soukup S, Ballard E: Chromosomal analysis of sixteen human rhabdomyosarcomas. *Cancer Res 48: 983-987, 1988*

Tomita T, Yasue M, Engelhard HH, Mclone DG,

Gonzalez-Crussi F, Bauer KD: Flow cytometric DNA analysis of medulloblastoma. Prognostic implication of aneuploidy. *Cancer 61: 744-749, 1988*

Turc-carel C, Philip I, Berger M-P, Philip T, Lenoir GM. Chromosome study of Ewing's sarcoma cell lines: consistency of a reciprocal translocation, t(11:22) (q 24: q 12). *Cancer Gent Cytogenet 12: 1-9, 1984*

Whang-Peng J, Triche TJ, Knutsen T, Miser J, Douglass EC, Israel MA: Chromosome translocation in peripheral neuroepithelioma. *N Engl J Med 311: 584-585, 1984*

Young JL, Miller RW: Incidence of malignant tumors in US children. *J Pediatr 86: 254-258, 1975*