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Flow Cytometric Determination of DNA Content in Renal Cell Carcinoma with Special Reference to Tumor Heterogeneity[1]

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=Abstract=**The DNA (deoxyribonucleic acid) content in 111 samples from 14 renal cell carcinomas (8 samples per one tumor specimen) was analyzed by flow cytometry to investigate DNA heterogeneity in renal cell carcinomas and DNA ploidy patterns were also compared with histologic grade and tumor stage. Of the 50 grade I and 22 grade II tumor samples, 46 (92%) and 14 (64%) were diploid respectively, but only 5 (13%) of 39 grade III & IV samples were diploid. This was statistically significant ($p=0.01$). Of the 9 $pT_2N_0N_0$ tumors, diploid were 4 (44%) and of 5 pT_3NanyM_0 tumors (4 $T_{3a}N_0M_0$, 1 $T_{3b}N_1M_0$), diploid was 1 (20%). No statistical correlation was made between tumor stage and ploidy status, because of the small number of specimens. Tumor heterogeneity was noted in 8 of 14 tumors (57%). Of the 6 homogeneous tumors, five were diploid and one was aneuploid. Of the 9 aneuploid tumors (64%), only one (7%) was homogeneously aneuploid and 8 (57%) were heterogeneously aneuploid. The probability of having an aneuploid sample by taking at random 1, 2, 3, 4 samples from a tumor specimen was 65%, 88%, 94% and 97%, respectively. In conclusion, renal cell carcinoma is heterogenous in terms of DNA ploidy. For flow cytometric evaluation at least 4 samples need to be analyzed to minimize the sampling error and obtain aneuploidy with 95% confidence in one tumor specimen.**

Key Words: *Flow cytometry, Tumor heterogeneity, Renal cell carcinoma*

INTRODUCTION

Concerning the prognosis of renal cell carcinoma, the pathological stage has been recognized as the most important factor(Selli *et al.* 1983). However, considerable histopathological and cytological variations are frequently found in renal cell carcinoma and it has been suggested that the histologically least differentiated and cytologically spindle-shaped or pleomorphic variants influence prognosis adversely (Skinner *et al.* 1971). But in the individual patient, the clinical outcome is difficult to predict. For example, among patients with similar histological and cytological features, some patients have a better prognosis and some have a worse prognosis. This phenomenon can be explained by tumor cell heterogeneity.

Despite the concept of clonal development of malignant neoplasms from a single cell, at the time of clinical presentation, parts of solid tumors show notable heterogeneity which has been explained by genetic instability of the original cell clone and selection of the most viable cell clones during

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the evolution from the original cell clone to an established tumor (Tribukait 1987).

By DNA measurement, cell clones with different DNA values may be detected and thus provide information of tumor heterogeneity. While the correlation of ploidy status with the clinical natural history of a specific tumor has been consistent in prostate and bladder malignancies, the results obtained with flow cytometry in renal cell carcinoma have been inconsistent and contradictory (Ljunberg *et al.* 1986; Kloppel *et al.* 1986; Otto *et al.* 1984; Schwabe *et al.* 1983; Rainwater *et al.* 1987). This may partly be explained by heterogeneity of ploidy status in a tumor. When two or more samples were analyzed from a single primary tumor, heterogeneity of ploidy status was observed in 30% by Currin *et al.* (1990).

This compelled us to evaluate the degree of heterogeneity concerning DNA ploidy in renal cell carcinoma specimens and find out optimal numbers of samples in one tumor specimen to minimize sampling error for flow cytometry. Additionally we attempted to compare DNA ploidy status with the histologic grade and tumor stage.

MATERIALS AND METHODS

Patient Identification

Flow cytometric analysis was performed on 14 patients who underwent radical nephrectomy between January and August 1990. Patient age ranged from 37 to 70 years with a mean age of 56 years. There were 11 men and 4 women. From each tumor specimen, eight tumor samples of about 5×5×5 mm. were taken as shown in Figure 1. The tissue samples were fixed in 0.1 M phosphate bufferred 4% formaldehyde (pH 7.2-7.4), embedded in paraffin. The tissue samples embedded in paraffin were divided into two parts, one for histologic examination and one for DNA flow cytometry.

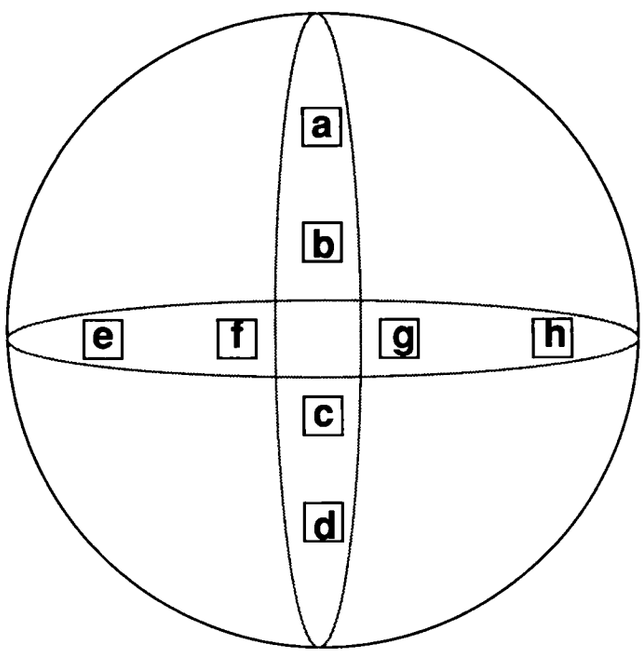

Fig. 1. Schematic illustration of sampling method.

Histopathologic Examination and Tumor Stage

All patients were evaluated with intravenous pyelogram, ultrasonography, abdominal computerized tomography and bone scan. All patients were pathologically staged according to TNM. Nine patients had pathological stage $pT_2N_0N_0$, 4 had $pT_{3a}N_0M_0$ and 1 had $pT_{3b}N_1M_0$. According to Skinner's method, we graded the renal cell carcinomas with respect first of all to nuclear atypia including the size of nucleoli. We divided tumor cell type into two groups. If a cytoplasm is microscopically clear, we designated those tumors as clear cell renal carcinomas. If a tumor does contain granular cells, spindle shaped or pleomorphic cells, we designated those tumors as non-clear cell renal carcinoma.

Preparation of Paraffin Embedded Archival Specimens

Sections(50 μm thick) from deparaffinized blocks were processed using a modification of a technique developed by Hedley and associates *et al.* (1983) as previously described by one of us (Currin *et al.* 1988). Briefly, deparaffinization was accomplished with 3 changes of 3 ml. Histoclear during 45 minutes with subsequent rehydration through sequential 3 ml, 10 minutes rinses of absolute, 95%, 70% and 50% ethanol. Specimens were then washed twice with distilled water and incubated in 3 ml 0.5% pepsin in 0.9% sodium chloride, pH 1.5, at 37℃ for 30 minutes with frequent vortex mixing. The resulting mononuclear suspension was filtered through a 41 μm nylon mesh and centrifuged at 1,500 revolutions per minute for 10 minutes. The pellet was washed twice with distilled water and ultimately resuspended in a staining solution

of 1 ml. 0.05% propidium iodide and 0.1% ribonuclease A in phosphate buffered saline. The nuclear count ranged from 5×10^5 to 3×10^6 per sample approximately.

Flow Cytometric Analysis

Flow cytometric analysis was performed on a Coulter Electronics EPICS 753 Cell Sorter using a 5 watt argon laser run at a wave length of 488 nm. DNA histograms were generated by counting 5×10^4 nuclei per sample. Flow cytometry was performed successfully on 111 samples from 14 tumor specimens. Only 1 sample was non-fluorescent. Fourteen deparaffinized normal kidney specimens from radical nephrectomy were used as controls for flow cytometric analysis. These samples were screened by light microscopy to assure absence of a pathologic condition. The mean G_2M peak in normals was 2.65 $\pm$ 1.03%. Therefore, tetraploid aneuploidy was defined as a cell population with greater than 5.7% of total nuclei in the G_2M peak (mean of normal G_2M plus 3 standard deviations of the mean). Nontetraploid aneuploidy was defined as the presence of any aberrent G_0/G_1 peak not located at the tetraploid position (4C $\pm$ 0.5 C). All data were analyzed statistically with standard parametric tests as indicated.

RESULTS

Heterogeneity of Ploidy Status in a Tumor Specimen

The mean coefficient of variation for all samples was 6.39 $\pm$ 1.42. No sample was excluded from analysis on the basis of a high coefficient of variation. Of 111 samples, 46 (41%) were aneuploidy. Of 46 aneuploidy samples, 27 (59%) were tetraploid aneuploid and 19 (41%) were nontetraploid aneuploid (Table 1). For purposes of data analysis, the tetraploid and nontetraploid aneuploid tu-

Table 1. DNA ploidy status from 111 tumor samples

Ploidy	No. samples (%)
Diploid	65 (59)
Aneuploid	46 (41)
Tetraploid	27
Nontetraploid	19
Total	111 (100)

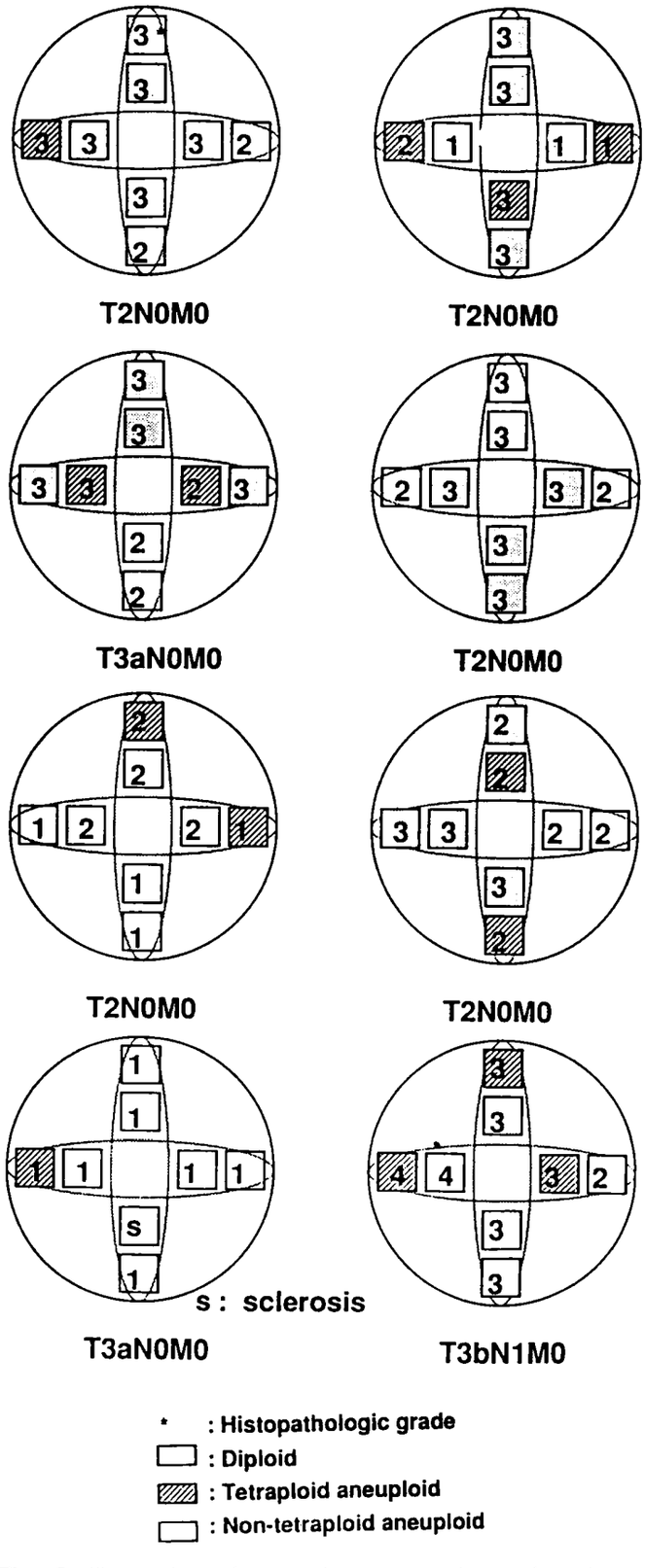

Fig. 2. Illustration of tumor heterogeneity as functions of DNA ploidy and histopathologic grade in 8 tumor specimens.

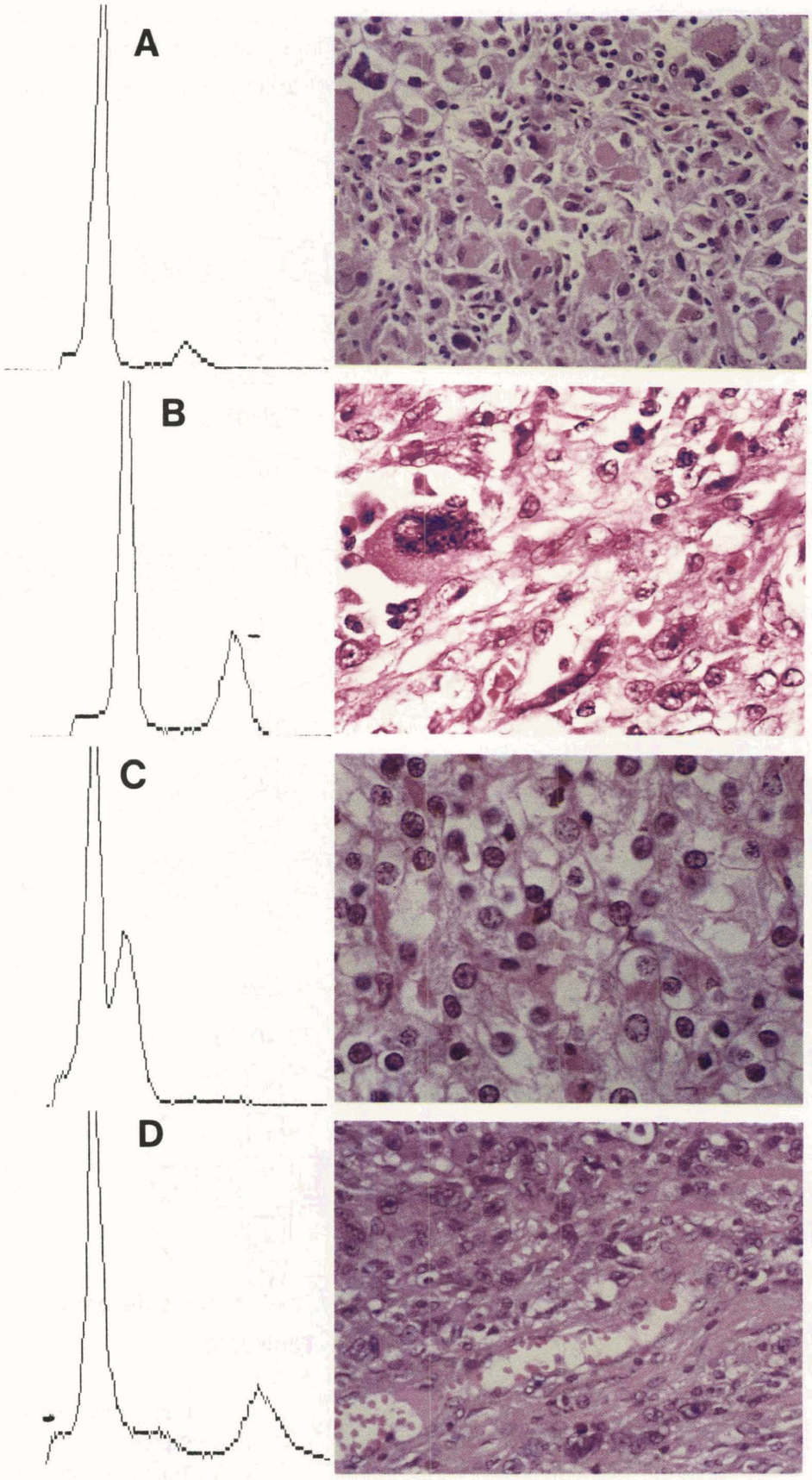

Fig. 3. DNA histogram and the corresponding histology in 1 patient (H & E, X 400). (A) diploid DNA content in a grade III tumor sample, (B) tetraploid aneuploid in a grade III tumor sample, (C) non-tetraploid aneuploid in a grade II tumor sample and (D) non-tetraploid aneuploid in a grade IV tumor sample. Note the tumor heterogeneity as functions of DNA ploidy and histopathologic grade in a tumor specimen.

mors were grouped as aneuploid tumors. If 8 samples from a tumor specimen were all diploid or aneuploid, we designated those as homogeneous diploid or aneuploid tumors, respectively. If samples from a tumor specimen showed a mixed pattern of diploidy and aneuploidy, we designated those as heterogeneous aneuploid tumors. Of 14 tumor specimens, 5 (36%) were homogeneously diploid and 1 was homogeneously aneuploid comprising of 43% of DNA homogeneity. In 8 patients (57%), heterogeneity in DNA content was noted within the primary tumor with diploid and aneuploid elements present. Fig. 2 illustrates heterogeneity as functions of ploidy and histopathologic grade in a tumor specimen in 8 heterogeneous aneuploid tumors. Different ploidies were noted in the same grade samples in a tumor specimen (Fig. 2) and also different tumor grades were noted in 2 of 6 samples showing the same ploidy in a tumor specimen. Fig. 3 shows representative DNA histograms and the corresponding histology in a tumor specimen from 1 patient.

Comparative Analysis of Tumor Grade and Ploidy Status

One hundred and eleven tumor samples were graded by a pathologist. Of 111 tumor samples, 50 were grade I, 22 were grade II, 35 were grade III and only 4 were grade IV. For the purposes of data analysis, grade III and IV tumor samples were grouped because the number of grade IV was only 4. Of the grade I tumor samples, 92% were diploid and of the grade II tumor samples, 64% were diploid compared to a 13% incidence of diploid for grades III and IV. This difference was statistically significant by chi-square analysis (Table 2).

Table 2. DNA ploidy according to the histopathologic grade

Grade	No. samples	Diploid (%)	Aneuploid (%)
I	50	46 (92)	4 (8)
II	22	14 (64)	8 (36)
III-IV	39	5 (13)	34 (87)
Total	111	65 (59)	46 (41)

$P < 0.01$

Comparative Analysis of Cell Type and Ploidy Status

Of 14 tumor specimens, 11 were clear cell renal carcinomas and 3 were non-clear cell renal carcinomas. Of 3 non-clear renal carcinomas, 2 specimens were mixed with clear and spindle cells and 1 specimen was granular cell renal carcinoma. All 3 non-clear cell carcinoma were aneuploid. No statistical difference could be made between the two groups because numbers were too small (Table 3).

Table 3. DNA Ploidy according to the cell type

Cell type	No. tumors	Diploid (%)	Aneuploid (%)
Clear	11	5 (45)	6 (55)
Non-clear	3	0 (0)	3 (100)

Comparative Analysis of Tumor Stage and Ploidy Status

Patients were grouped into 2 categories on the basis of TNM staging (Table 4). PT_2 consisted of 9 patients of whom 4 (44%) had diploid tumors. PT_3 consisted of 5 patients of whom 1 (20%) had diploid tumor. Although there was an increased incidence of diploid tumor in pT_2 group, the small number of patients with pT_3 tumors made statistical assessment inappropriate.

Table 4. DNA ploidy according to the stage (TNM)

Stage	No. tumors	Diploid (%)	Aneuploid (%)
pT_2	9	4 (44)	5 (56)
*pT_3	5	1 (20)	4 (80)

* $T_{3a}N_0M_0$: 4 cases, $T_{3b}N_1M_0$: 1 case

Determination of Appropriate Sample Numbers for Flow Cytometry in a Tumor Specimen

Heterogeneous aneuploid tumors were noted in 8 specimens and homogeneous aneuploid tumor was noted in 1 specimen. When one takes at random 1, 2, 3, and 4 samples from a tumor with aneuploidy, the probability of finding an aneuploid sample was 65%, 88%, 94% and 97%, respectively. Therefore at least 4 samples need to be ana-

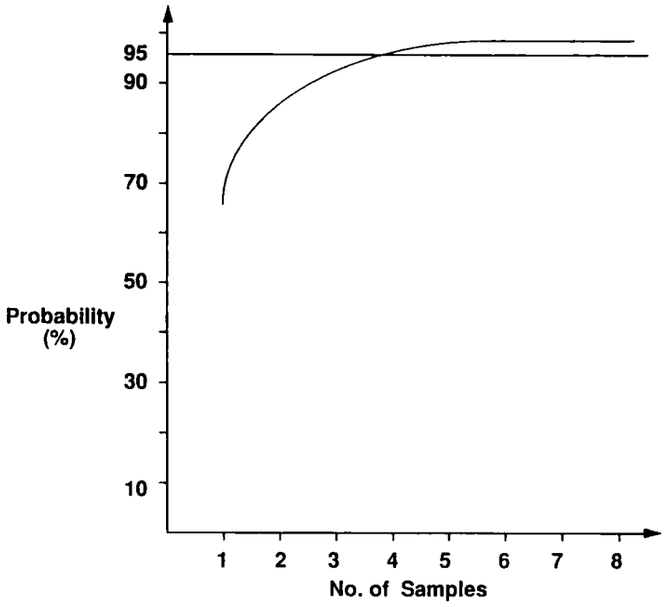

Fig. 4. Probability of obtaining aneuploid tumor sample by taking 1 to 8 samples from 9 aneuploid tumors.

lyzed to minimize the sampling error and obtain aneuploidy with 95% confidence in one tumor specimen. This calculation was made by using the concept of binomial distribution (Fig. 4).

DISCUSSION

Among the parameters that have been analyzed with respect to the prognosis of renal cell carcinoma, TNM staging and tumor histopathologic grade are recognized to be the most critical prognostic parameters in renal cell carcinoma. Because of the unpredictable natural history of surgically treated renal cell carcinoma, there is a continuing search for a methodology that may afford greater accuracy in assessing tumor biology in an individual patient. Among the alternative methods to assess the malignant potential of a tumor is flow cytometry, an innovative technique of tumor cell analysis providing objective information regarding quantitative aberration of deoxyribonucleic acid content. Many recent articles have reported a strong correlation between an abnormal DNA content, and high grade, stage and poor clinical outcome in various genitourinary malignancies. For bladder and prostatic carcinomas, a strong correlation between the DNA content and the biological behavior of the tumor has been noted consistently (Tribukait 1987; Lee *et al.* 1988; Schutte *et al.* 1985; Barlogie *et al.* 1983; Frankfurt *et al.* 1985; Hedley *et al.* 1985). On the other hand, far less consistency and a frank discordance between the DNA content and the biological behavoir of renal cell carcinoma has also been noted. Differences in the incidence of aneuploidy, the correlation of ploidy status and tumor stage and the prognostic value of ploidy analysis in predicting survival in renal cell carcinoma are all controversial issues.

Linden *et al.* (1980) correlated the cellular DNA content of renal cell carcinomas with histopathologic grade and clinical prognosis, reporting that high grade renal cell carcinomas often had aneuploidy stemline and that cancer with aneuploid stemline were much more likely to recur than ones consisting only of diploid stemlines. However, other investigators have found no correlation between aneuploidy and the high stage or grade of renal cell carcinoma (Kloppel *et al.* 1986; Schwabe *et al.* 1983). The recent report by Ljunberg *et al.* (1985) showed that clones with differing DNA content were found in nearly half of the 25 renal cell carcinomas studied. This DNA heterogeneity within neoplasms may account for some of the discrepancies between the results of earlier investigators.

According to our study, aneuploid stem line was found in 9 of 14 (64%) tumor specimens (Table 3, 4). Our findings are consistent with most reports in the literature regarding the frequency of aneuploidy, ranging from 45% to 74% (Friedlander *et al.* 1984; Chin *et al.* 1985). Our findings are thought to be more accurate than others because most investigators used a single tumor sample for DNA analysis. When we analyzed DNA content in 111 samples from 14 tumors (8 samples per each specimen), the frequency of aneuploidy decreased to 41% because there were 5 homogeneous diploid tumors (40 samples) (Table 1).

In comparative analysis between tumor grade and ploidy status from 111 samples, the frequency of aneuploidy increases as tumor grade increases. A concordant relationship between tumor grade and ploidy was noted in a statistically significant manner (Table 2). This finding was similar to those of other investigators (Tribukait 1987; Ljunberg

et al. 1986). It is conceivable that increase in nuclear size as an important parameter of nuclear grading is most likely paralleled by an increased DNA content resulting in aneuploidy (Kloppel *et al.* 1986).

An important focus in this study is an evaluation of tumor heterogeneity in terms of tumor grade and particularly DNA ploidy status. Heterogeneity of tumors seems to be of main clinical importance since it has been demonstrated that treatment must be directed against separate coexisting cell clones in many malignancies. From studies of carcinoma in situ of the bladder by Tribukait (1987), only tumors with more than one hyperdiploid clone progress and become invasive. Subpopulations of high proliferative activity have been demonstrated in the area near the invasive border.

By DNA measurement, cell clones with different DNA values may be detected and thus provide information of tumor heterogeneity. Since considerable morphologic variations are frequently found in renal cell carcimoma, it was interesting to analyze the DNA content in different parts of the primary tumor. Concerning heterogeneity of tumor grade, we found that approximately 33% (2 of 6 tumor specimens) in our study showed considerable variation in a tumor histology which was composed of more than one grade in the same tumor which showed homogeneous DNA content.

Kloppel *et al.* (1986) reported approximately 10% of intratumoral heterogeneity of tumor grade. The DNA heterogeneity confirmed by us within the primary tumor specimens deserves particular emphasis. Although early reports suggested that there was little variation in ploidy status at different sites within the primary tumor specimens, our findings support the prevailing view of more recent literature that pronounced heterogeneity of ploidy status commonly exists in renal cell carcinoma. In our previous article, we reported the heterogeneity of ploidy in primary tumors, discordance in ploidy between primary and metastatic tumors, and high incidence of diploidy at metastatic sites (Currin *et al.* 1990).

Ljunberg *et al.* (1985) studied 25 patients with renal cell carcinomas, from whom at least 8 samples from each tumor specimen were analyzed, and found heterogenity in DNA content in 44%. In the present study, there was a 57% incidence of heterogeneity. From the pragmatic clinical standpoint this means that sampling only at 1 site may be problematical, markedly limiting the general clinical use of this approach.

Nowell (1976) emphasized that genetic instability is an innate characteristic of many malignant tumors and this may result in substantial heterogeneity for many phenotypes expressed. That DNA content heterogeneity is such a prominent feature in a multifocal analysis of DNA content in primary tumor specimens may indicate that phenotypic heterogeneity for many expressed molecular characteristics such as antigen expression, receptor expression, enzyme levels and so forth, could be a common phenomenon in renal cell carcinoma.

In conclusion, renal cell carcinoma shows prominent intratumoral heterogeneity in terms of DNA ploidy. For DNA flow cytometry in renal cell carcinoma, at least 4 samples from one tumor specimen need to be analyzed to minimize sampling error, and achieve 95% confidence of having aneuploid cell clones. Since a correlation between tumor grade and ploidy status was noted, it is suggested that detection of aneuploid stemline obtained from multiple samples from a specimen is correlated with a higher incidence of disease progression and decreased survival.

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=국 문 초 록=

신세포암에서 종양의 이질성을 고려한 DNA Flow Cytometry 분석

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저자들은 14례의 신세포암 환자에서 총 111개의 종양조직을 얻어 DNA flow cytometry를 시행하여 종양의 이질성에 관한 분석을 시행하고 아울러 그 결과를 조직학적 분화도 및 종양기와 비교하였다. 50개의 분화도 I 및 22개의 분화도도 II의 표본 중 46개(92%) 및 14개(64%)가 diploid인 반면 39개의 분화도 III과 IV의 표본 중에서는 5개(13%)가 aneuploid로 두 군간에는 통계적으로로 유의한 차이가 있었다(P-value=0.01). 9례의 $pT_2N_0N_0$ 종양에서 diploid는 4(44%)례, 5례의 pT_3NanyM_0(4 $T_{3a}N_0M_0$, 1 $T_{3b}N_1M_0$) 종양에서는 diploid가 1(20%)례였고 표본의 수가 적어 종양기와 ploidy와의 통계적인 유의성은 구하지 못하였다. 종양의 이질성은 14례의 종양 중 8례(57%)에서 있었고, 6례의 동질적인 종양 중 5례는 동질적인 diploid, 나머지 1례는 동질적인 aneuploid였다. 9례(64%)의 aneuploid 종양 중 1례(7%)만 동질적인 aneuploid였고 나머지 8례(57%)의 종양은 이질적인 aneuploid였다. 1개의 종양에서 무작위로 추출한 1개, 2개, 3개, 4개의 조직표본에서 aneuploid를 가질 확률은 각각 65%, 88%, 94% 및 97%였다. 결론적으로, 신세포암은 DNA ploidy라는 관점에서 보면 이질적이며 flow cytometry 분석에 있어서 종양조직이 95% 이상의 확률로 aneuploidy를 갖기 위해서는 최소한 한 종양조직에서 4개의 조직표본을 얻어야 할 것으로 사료된다.