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Influence of vinblastine on DNA parameters and multidrug resistance in renal cell carcinoma in vitro

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Abstract The purpose of this study was to investigate how the vinca alkaloid vinblastine influences DNA parameters and the mechanisms of multidrug resistance in renal cell carcinoma. After exposing cell cultures of human renal carcinoma to progressively increasing concentrations of vinblastine the cell lines were examined by flow cytometric DNA analysis to assess the S-phase and G_2/M-phase fraction and by a modified MTT assay. It was shown that the exposed cells became P-glycoprotein-positive by staining the cells with a monoclonal antibody (JSB-1). The flow cytometric analysis revealed, with prolonged vinblastine exposure, correlated increases in the S-phase and G_2/M-phase fractions ($P = 0.0001$). When vinblastine-free medium was used for culturing, the changed DNA characteristics returned to their original values. Comparing the DNA parameters with the IC_{50} (concentration when cell growth is inhibited by 50%) we found a strong correlation between these parameters ($P = 0.0001$). In conclusion, DNA analysis of long-term vinblastine exposure may provide insight into events leading to multidrug resistance. Furthermore, analysis of the DNA profile might also be an important investigation before planning therapy with vinblastine for renal cell carcinoma.

Key words Renal cell carcinoma · Vinblastine · Therapy · Multidrug resistance · DNA parameters · MTT assay

Introduction

The biological behavior of renal cell carcinoma must be taken into consideration when planning therapy. Multidrug chemotherapy, with an average response rate of 15%, has not played a major role in the therapy of renal carcinoma. One chemotherapeutic agent commonly used for therapy is the vinca alkaloid vinblastine [2, 11, 14], which leads to remission in 20% of cases [21]. It is known to bind specifically to tubulin and to block cells in mitosis [22]. Other effects of vinblastine on intracellular mechanisms are also presumed. The best-investigated alteration caused by vinblastine in multidrug-resistant cells is the overexpression of membrane-linked P-glycoprotein. P-glycoprotein acts as an ATP-ase-dependent efflux pump [7, 9, 25]. The expression of P-glycoprotein has been identified as an adverse prognostic factor [20]. The efficiency of drug efflux can be inhibited by several agents, which act competitively [19]. A variety of compounds, called chemosensitizers, inhibit the function of P-glycoprotein [8, 16]. The carcinogenic and mutagenic effect of vinblastine, which is shown in an increased rate of ploidy alteration, can influence the malignancy of surviving cells during chemotherapy. Vinblastine is able to influence the cells in such a way that a more malignant, e.g. tetraploid, phenotype is induced due to DNA duplication under drug selection. The individual biological behavior is expressed in altered grading and DNA status. An influence of the percentage of S-phase fraction on survival has also been reported [12, 26, 29].

Methods

Six cell lines of renal cell carcinoma (BN 4, 9, 12 ,13, 17 and 18) cultured from tumor samples of patients in our department were used. The cell lines were from tumors of different grades (G1–G3) and were incubated for 10 months with vinblastine. The dose of vinblastine was increased from 0.5 ng/ml to 400 ng/ml (0.5, 1, 5, 10, 50, 100, 200 and 400 ng/ml) after every third passage. With an estimated confluence of 70% had been reached, the cells were disaggregated and placed in new culture flasks. Immunostaining was used to evaluate multidrug resistance. Cells were studied with a modified MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [23] and with flow cytometry before and after increases in vinblastine.

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Cytotoxicity was assessed using an MTT assay [4]. The cells were incubated for 2 h in vinblastine concentrations ranging from 0.05 to 4.0 μg/ml. The cytotoxicity and level of chemoresistance to vinblastine were evaluated by assessing the concentration at which vinblastine inhibited cellular growth by 50% (IC_{50}) [25].

The DNA index and the proportions of cells in the different phases of mitosis were calculated on the basis of the differences in fluorescence after the sample was measured in ICP 11/22 (Phywe, Göttingen, Germany) [17]. Results were calculated using the methods described by Severin et al. [28] and Büchner et al. [6].

Immunostaining the cells with the monoclonal antibody JSB-1 was used to evaluate whether the cell lines expressed P-glycoproteins before or only after long-term vinblastine exposure [10, 31]. On fluorescence microscopy the cell lines analyzed were called positive if ≥10% of the cells showed a positive signal, defined as distinct staining of the nuclear membrane or Golgi complex or at least diffuse staining of the cytoplasmic membrane.

Statistical analysis

The correlation coefficient, [ρ], was calculated with repeated observations [3]. Taking into consideration the different biological behavior of the cell lines, the methods of Blad and Altman [3] were applied. The analysis was done by the statistical program package SAS [27] and [*P* values for the test of no correlation were reported. *P* values < 0.05 were interpreted as a correlation differing significantly from 0.

Results

When analyzing the development of chemoresistance to vinblastine in renal carcinoma cells the IC_{50} rose slowly in the first steps of increased vinblastine concentration in culture medium. Above a concentration of 50 ng/ml the IC_{50} increased rapidly, as shown by the results of MTT assay. The development of drug resistance to vinblastine is shown in Fig. 1. The examination of P-glycoprotein expression showed that the untreated cell lines were P-glycoprotein-negative, although occasional positive cells could be seen before exposure to vinblastine. After exposing the cells to vinblastine the immunostaining with JSB-1 revealed that the cells became P-glycoprotein-positive (Fig. 2).

Following an increase in vinblastine dose, cell proliferation decreased (average 7 days). In the next passage, however, cell proliferation returned to former levels (2–3 days). The morphology of the cells was characterized by an inhomogeneous appearance after each dose increase: differences in size, granulation, nucleocytoplasmic ratio and cell detritus were found. The appearance of the cells, however, normalized in the following passages at the same concentration of vinblastine. Similar observations were made regarding the viability of the cells: after an increase in dose the number of viable cells, as determined by trypan blue exclusion, decreased on average to 75%, but in further passages the coefficient of viability returned to about 97%.

To evaluate how the DNA parameters of previously vinblastine-treated cells react on exposure to vinblastine-free medium, flow cytometry was performed before treatment, during treatment and after vinblastine exposure. Figure 3 shows the flow cytometric changes

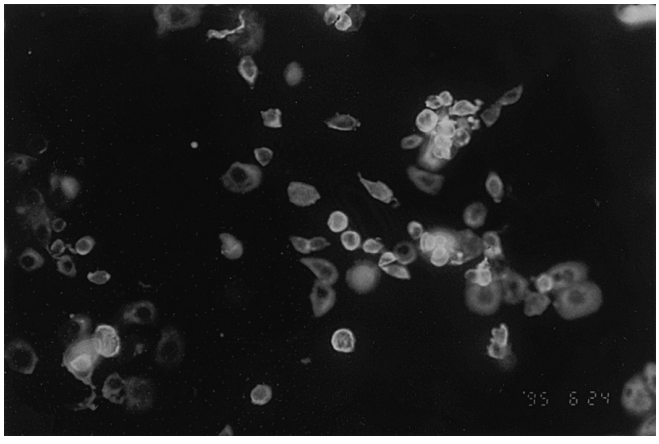

Fig. 2 Immunostaining with the monoclonal antibody, JSB-1 against P-glycoprotein. Under long-term vinblastine treatment the renal carcinoma cells now express P-glycoprotein

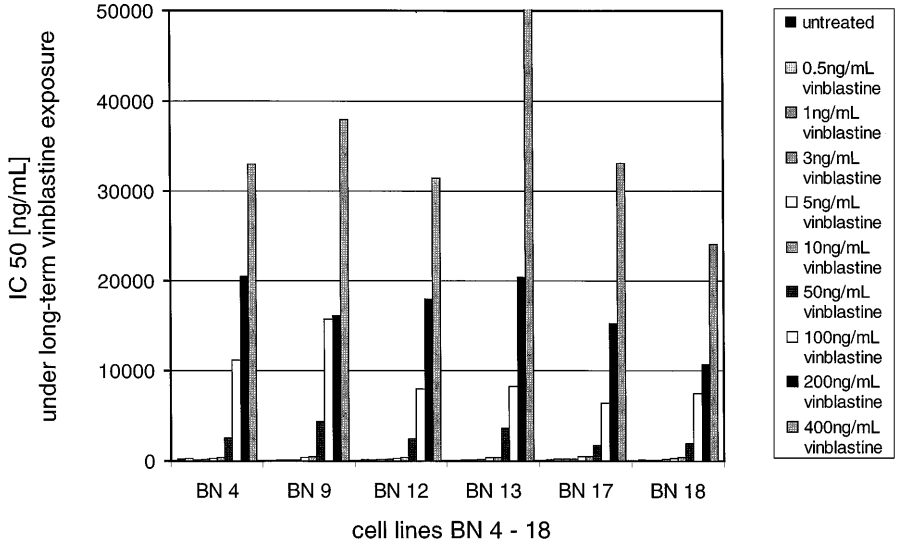

Fig. 1 IC_{50} values of six renal cell carcinoma cell lines incubated with increasing concentrations of vinblastine (untreated cells; cells at 5 ng/ml, at 400 ng/ml vinblastine). A rise in IC_{50} reflects an increase in maximal tolerated drug concentration of vinblastine in the culture medium

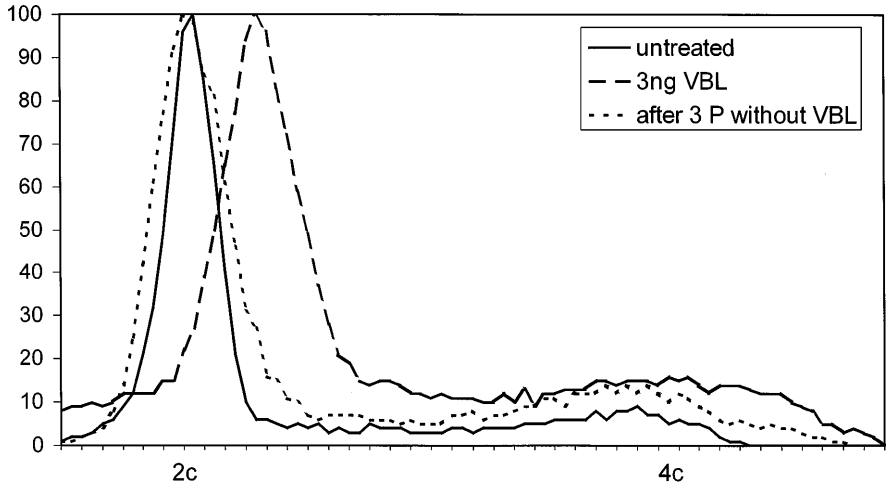

Fig. 3 Histograms of cell line BN 4 before treatment with vinblastine (*VBL*), during treatment (vinblastine at 3 ng/ml), and after three passages (*P*) without vinblastine following incubation with vinblastine (3 ng/ml). When vinblastine-free medium was used for culturing, a return of DNA content and a decrease in the G_2/M- and S-phase fractions were seen

Table 1 Changes in the G_2/M-phase fraction with increase in vinblastine concentration for development of multidrug-resistance. Low-dose versus high-dose results demonstrate the influence of drug exposure on G_2/M-phase cells

Cell line	Culture medium vinblastine concentration *or* change in G_2/M-phase fraction					
	0	5 ng/ml	ΔG_2/M	100 ng/ml	400 ng/ml	ΔG_2/M
BN 4	6.3%	15.0%	8.7%	20.6%	24.0%	3.4%
BN 9	10.1%	14.6%	4.5%	17.0%	17.2%	0.2%
BN 12	7.0%	13.0%	6.0%	16.0%	21.7%	5.7%
BN 13	14.0%	15.7%	1.7%	20.8%	22.3%	1.5%
BN 17	14.0%	18.5%	4.5%	19.0%	19.9%	0.9%
BN 18	8.8%	14.7%	5.9%	22.0%	23.8%	1.8%

resulting from vinblastine exposure of cell line BN 12. Similar behavior was seen in all other cell lines. In cells treated with 3 ng/ml for three passages, the proportion of cells in G_2/M-phase increased by 4.2%, while the S-phase fraction rose by 12.5%. After exposure to vinblastine-free medium for three passages, the G_2/M-phase fraction shows an increase of 2.8% and the S-phase fraction of 3.4% compared with the untreated cells. The DNA content, which is determined by DNA index, changed with vinblastine exposure up to 0.75. After three passages in vinblastine-free medium, DNA content returned to its initial value.

The observation of alterations in the DNA profile showed that the proportion of the cell-cycle phases changed with the tolerated drug concentration on exposure to vinblastine. We found that the proportion of the G_2/M-phase increased (average 10.8% vs 15.9%) at the start of vinblastine exposure. In the following dose steps, the increase in G_2/M cells was reduced (Table 1). By increasing vinblastine concentration, the S-phase fraction rose as well. Analyzing both increasing S- and G_2/M-phase fractions a correlation between the S- and G_2/M-phases was observed for all concentrations ($\rho = 0.633$, $P = 0.0001$) (Fig. 4). Comparing the DNA parameters with the IC_{50} we found a correlation of $\rho = 0.843$ (IC_{50} vs S-phase fraction, $P = 0.0001$) and $\rho = 0.586$ (IC_{50} vs G_2/M-phase fraction, $P = 0.0001$).

Discussion

The conditions under which drug resistance is acquired during vinblastine exposure are poorly understood. Both selection of primary P-glycoprotein-positive cell populations and vinblastine induction of P-glycoprotein expression in primary P-glycoprotein-negative cells have been suggested to account for the development of vinblastine-resistant phenotypes [1, 18, 30]. The influence of topoisomerase II activity and glutathione-*S*-transferase [13] level is not essential for multidrug resistance against vinca alkaloids [1]. Atypical drug efflux mechanisms might also participate in the loss of drug-induced cytotoxic efficiency of vinblastine [1]. In the present study, we investigated the effects of vinblastine on multidrug resistance development in vitro and on DNA parameters. A tolerated concentration of 50 ng/ml leads to an increase in IC_{50} by a factor of 9–28 compared with cells treated with vinblastine-free medium only. In the early concentration steps (0.5–5 ng/ml) the population doubling time increases by a factor of 1.5. During the first days of incubation the cells with the next highest concentration of vinblastine had almost no microscopically observed proliferation activity. In addition, we did not find substantial changes in the number of vital cells during this period. Thus, the increase in multidrug resistance appears to be due to a change in metabolism, which is indicated by resistance during vinblastine

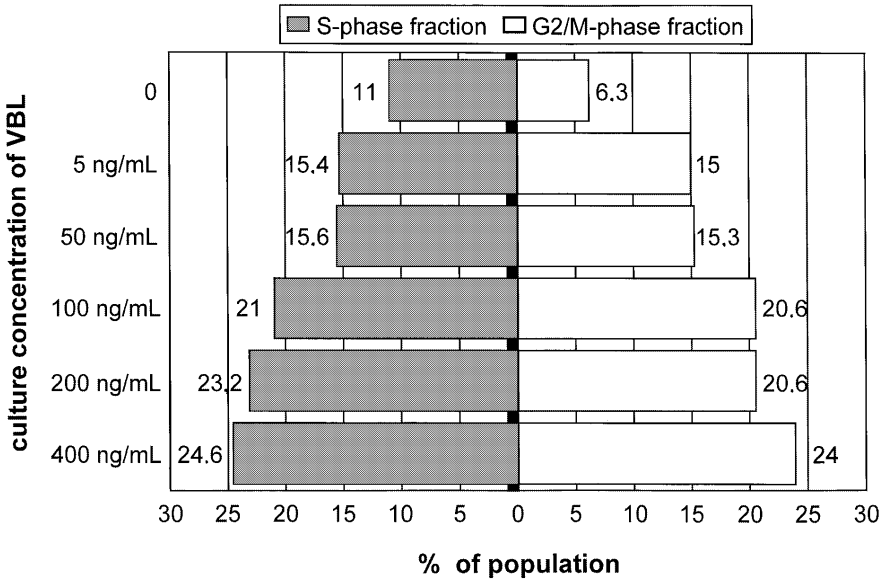

Fig. 4 Fractions of G_2/M- and S-phases in cell line BN 4

treatment, rather than to selection of resistant subpopulations. The course of multidrug resistance development shows that it is dose-dependent. Doses capable of reducing cell proliferation also affect multidrug resistance induction, whereas incubation with low doses of drug induces only slight increases in multidrug resistance. Our data suggest that the development of multidrug resistance depends on the concentration and duration of vinblastine exposure.

In the part of this study where cells were exposed to vinblastine and afterwards to vinblastine-free medium for three passages, the DNA index returned to the former value of the untreated cells. Also the other DNA parameters returned at least partly to former values. The slightly extended base of the G_1 peak after the final exposure to vinblastine-free medium might document the vinblastine-induced damage to the cells, although there is no explicit evidence for this hypothesis. Another possible explanation for these alterations is that the vinca alkaloid has induced a vinblastine-selected cell line with apparent changes in the cell cycle.

Briffod et al. [5] reported that diploid tumors are resistant to chemotherapy, whereas cytomorphological changes during treatment indicate chemosensitivity. We investigated DNA content and the changes in cell-cycle proportions during vinblastine exposure and showed that the maximal tolerated vinblastine concentration in the culture medium is strongly correlated with an increase in the S- and G_2/M-phase fraction. DNA is often the target of cytostatic agents; the vinca alkaloids react with the microtubuli of the spindle during the metaphase of mitosis. But other mechanisms of vinblastine action might exist. The increase in the G_2/M fraction is consistent with multidrug resistance development. This finding might be related to the fact that – despite the efficient drug efflux by P-glycoprotein – vinblastine is still able to enter the cells and thereby to influence the cell cycle. Possibly the pumping capacity of P-glycoprotein is not able to expel the agent completely, and vinblastine can induce a block in cells that are not adapted to the new conditions.

During vinblastine treatment the S-phase fraction as an indicator of cell kinetics increased substantially. There are two possible explanations: the increased dose and biological mechanisms induce a higher proliferation rate as compensation, or the DNA synthesis time for duplication is prolonged by vinblastine treatment. The effect seems to be that the cells need more time to complete the cell cycle and stay longer in S-phase. In our study, however, the required time for outgrowth was prolonged only in the first passage after the increase in vinblastine concentration, whereas in the next two passages the growth time declined to the previous value. The results reflect an adaptation to the new condition.

Our observations agree with reports of a poor response rate in tumors with a low S-phase fraction [24]. The cell line BN 17, with the lowest proliferation fraction, displayed moderate adaptation to vinblastine; the initial IC_{50}, however, was relatively high. The S-phase fraction of tumors is used to predict clinical response to chemotherapy, whereas DNA content is not an indicator of chemosensitivity [26]. Spyratos et al. [29] reported an increased S-phase fraction in samples of tumor tissue after each course of chemotherapy. The G_2/M-phase fraction was not calculated. These results agree well with our in vitro findings. The S-phase fraction on its own provides only a partial view of tumor cell proliferation and does not predict the duration of the cell cycle, especially of the S-phase. From a practical point of view such studies are demanding, and the complexity of the drug action sometimes makes interpretation impossible.

This present study showed two things. First, it has provided an insight into how the vinca alkaloid vinblastine influences the DNA profile and the development of chemoresistance and might be a basis for further studies on the molecular effects of drugs on renal cell

carcinoma. Second, we suggest it may be of use to calculate the S- and G_2/M-phase fraction in patients when planning treatment with vinblastine. In this way, the response and possible development of chemoresistance can be estimated before beginning treatment. If the prospect of failure is too high the treating physician should consider an alternative therapy.

References

1. Beck WT (1990) Mechanism of multidrug-resistance in human tumor cells: the roles of P-glycoprotein, DNA topoisomerase II, and other factors. Cancer Treat Rev 17 [Suppl A]:11
2. Bergerat JP, Herbrecht R, Duflour P, Jacqmin D, Bollak C, Prevot G, Bailly G, De Garis S, Juraschek F, Oberling F (1988) Combination of recombinant interferon-alpha-2a and vinblastine in advanced renal cell cancer. Cancer 62:2320
3. Bland JM, Altman DG (1995) Calculating correlation coefficients with repeated observations. I. Correlation within subjects. BMJ 310: 446
4. Bosanquet AG (1994) Short-term in vitro drug sensitivity tests for cancer chemotherapy: a summary of correlations of test result with both patient response and survival. FORUM Trends Exp Clin Med 4: 179
5. Briffod M, Spyratos F, Tubiana-Hulin M , Pallud C, Mayras C, Filleul A, et al (1989) Sequential cytopunctures during preoperative chemotherapy for primary breast carcinoma: cytomorphologic changes, initial tumor ploidy, and tumor regression. Cancer 63:631
6. Büchner T, Dittrich W, Göhde W (1971) Die Impulszytophotometrie in der hämatologischen Cytologie. Klin Wochenschr 49:1090
7. Cordon-Cardo C, Lloyd FO, Finstad CL (1986) Immunoanatomic distribution of blood group antigens in the human urinary tract: influence of secretor status. Lab Invest 55:444
8. Damiani D, Michieli M, Michelutti A, Melli C, Cerno M, Baccarani M (1993) D-Verapamil downmodulates p170-associated resistance to doxorubicin, daunarubicin and idarubicin. Anticancer Drugs 4:173
9. Dutt A, Heath LA, Nelson JA (1994) P-glycoprotein and organic cation secretion by the mammalian kidney. J Pharmacol Exp Ther 269:1254
10. Efferth T, Volm M (1992) Immunocytochemical detection of oncoproteins in animal and human tumor lines with acquired or inherent multidrug resistance. Cancer Detect Prev 16: 237
11. Fossa SD, De Garis ST, Heier MS, Flokkmann A, Lien HH, Salveson A, et al (1986) Recombinant interferon-alpha-2a with or without vinblastine in metastatic renal cell carcinoma. Cancer 57:1700
12. Giaretti W, Santi L (1990) Tumor progression by DNA flow cytometry in human colorectal cancer. Int J Cancer 19:597
13. Hoban PR, Robson CN, Davies SM, Hall AG, Cattan AR, Hickson ID, et al (1992) Reduced topoisomerase II and elevated alpha class glutathione-*S*-transferase expression in a multidrug resistant CHO cell line highly cross-resistant to mitomycin C. Biochem Pharmacol 43:685
14. Hrusheksy BY, Murphy GP (1977) Current studies of the therapy of advanced renal cell carcinoma. J Surg Oncol 9:277
15. Kleinhans G (1989) DNS-Impulszytophotometrie in der urologischen Onkologie. Zuckschwerdt, Muncihen
16. Leyland-Jones B, Dalton W, Fisher GA, Sikic BI (1993) Reversal of multidrug resistance to cancer chemotherapy. Cancer 72:3384
17. Maier G, Heissler HE, Blech M, Schroter W (1988) DNA-Profile, Rezidivrate und Progression beim oberflachlichen G2-Karzinom der Harnblase. Urologe A 27:173
18. Marino PA, Gottesman MM, Pastan I (1990) Regulation of multidrug-resistance genein regenerating rat liver. Cell Growth Differ 1:57
19. Michelson S, Slate D (1994) A mathematical model for the inhibition of the multidrug resistance-associated P-glycoprotein pump. Bull Math Biol 56:207
20. Mickisch GH, Schroeder FH (1994) From laboratory expertise to clinical practice: multidrug-resistance-based gene therapy becomes available for urologists. World J Urol 12:104
21. Mickisch G, Roehrich K, Koessig J, Forster S, Tschada R, Alken P (1990) Mechanisms and modulation of multidrug resistance in primary human renal cell carcinoma. J Urol 144: 755
22. Miller BM, Zitzelsberger HF, Weier HU, Adler ID (1991) Classification of micronuclei in murine erythrocytes: immunofluorescent staining using CREST antibodies compared to in situ hybridization with biotinylated gamma satellite DNA. Mutagenesis 6:297
23. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55
24. Osborne CK (1989) DNA flow cytometry in early breast cancer: a step in the right direction (editorial). J Natl Cancer Inst 81:1344
25. Pauwels O, Kiss R (1991) Digital morphonuclear analyses of sensitive versus resistant neoplastic cells to vinca-alkaloid, alkylating and intercalating drugs. Cytometry 12:388
26. Remvikos Y, Beuzeboc P, Zadjela A, Voillemot M, Magdelénat H, Pouillart P (1989) Correlation of pretreatment proliferative activity with the response of cytotoxic chemotherapy. J Natl Cancer Inst 81:1383
27. SAS Institute (1987) SAS user's guide, version 6. SAS Institute, Cary, NC
28. Severin E, Meier EM, Willers R (1984) Flow cytometric analysis of mouse hepatocyte ploidy. I. Preparative and mathematical protocol. Cell Tissue Res 238:643
29. Spyratos F, Briffod M, Tubiana-Hulin M, Andrieu C, Mayras C, Pallud C, et al (1992) Sequential cytopunctures during preoperative chemotherapy for primary breast carcinoma. Cancer 69:470
30. Thorgeirsson SS, Huber BE, Sorrell S, Fojo A, Pastan I, Gottesman MM (1986) Expression of the multidrug resistant gene in hepatocarcinogenesis and regenerating rat liver. Science 236:1120
31. Volm M, Kastel M, Mattern J, Efferth T (1993) Expression of resistance factors (P-glycoprotein, glutathione *S*-transferase-pi, and topoisomerase II) and their interrelationship to proto-oncogene products in renal cell carcinomas. Cancer 71: 3981