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Cell proliferation in renal cell carcinoma – a comparative study of cell kinetic methods

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Abstract Four different methods of assessing cell proliferation in renal cell carcinoma were compared in a total of 136 samples to analyze their degree of agreement and usefulness. The methods compared were flow cytometric S-phase (S-FCM) analysis, proliferating cell nuclear antigen expression detected by immunohistochemistry (PCNA-IHC), in vivo iododeoxyuridine incorporation analyzed with immunohistochemistry (IdUrd-IHC), and flow cytometry (IdUrd-FCM). The mean S-FCM fraction was 5.9%, compared with a mean PCNA-IHC labeling index of 4.7%. The mean labeling indices obtained by IdUrd-IHC and IdUrd-FCM were 1.2% and 1.7%, respectively. The four methods correlated well with each other. When the methods were compared according to Bland and Altman, good agreement was shown. A statistically significant difference in proliferation between diploid and aneuploid tumor samples was found with all methods ($P < 0.001$). The results showed that the four different methods provided comparable information on proliferative activity, although different cell cycle compartments were monitored.

Key words Renal cell carcinoma · Cell proliferation · S-phase · PCNA · Iododeoxyuridine incorporation

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Introduction

Renal cell carcinoma is a highly unpredictable neoplasm, and prognostic parameters identifying patients at risk of recurrent disease and metastatic probability need to be established. Tumor stage, DNA ploidy and histopathologic grade are currently considered to be the most reliable prognostic parameters [16]. Cell proliferation is a fundamental biological process known to influence tumor progression and prognosis [4], and in recent years there has been considerable interest in finding reliable methods to assess cell proliferation.

Cell proliferation has been assessed in renal cell carcinoma using in vitro ^{3}H-thymidine labeling and autoradiographic evaluation [10]. This method is, however, unsuitable for clinical use since it requires administration of radiolabeled thymidine and extensive laboratory work. A commonly used method to measure proliferative activity is the determination of the proportion of DNA-synthesizing cells, the S-phase fraction, by flow cytometric DNA histogram evaluation (S-FCM) [2]. A more complete cell kinetic picture can be obtained by measuring in vivo incorporation of a non-radioactive thymidine analog, such as iododeoxyuridine (IdUrd), in cells actively replicating DNA. The IdUrd-labeled cells can be detected by specific antibodies using either immunohistochemistry (IHC) or flow cytometry [19]. Another approach to acquiring information on cell proliferation is the use of specific antibodies that recognize proteins with cell cycle phase-related expression, such as proliferating cell nuclear antigen (PCNA) [13, 18]. PCNA is a well-defined nuclear antigen, the expression of which is associated with cells in active cycle and the method can furthermore be carried out on conventionally formalin-fixed and paraffin-embedded tissue.

The aim of this study was to compare cell kinetic data obtained by four different methods and to analyze their degree of agreement in order to evaluate proliferative activity in renal cell carcinoma.

Materials and methods

Tumors

Surgical tumor samples were obtained from patients with renal cell carcinoma operated upon with perifascial nephrectomy during the period 1986 through 1991, at the Department of Urology, University Hospital, Umeå, Sweden. The specimens were obtained by schematically taken biopsies from the kidney with multiple tumor samples and one kidney cortex sample [8]. All samples included in the study were analyzed for DNA ploidy and had at least two of the following parameters analyzed: S-FCM, PCNA-IHC, IdUrd-IHC and IdUrd-FCM. The compared data correspond to the different techniques analyzed from the same tumor sample. Some of the cell kinetic data on which this comparative study was based have been published previously [7–9, 12].

DNA ploidy and flow cytometric S-phase analysis

The method for flow cytometric DNA analysis has been outlined previously [11]. Briefly, the fresh samples were minced and stained using a propidium iodide solution and run in a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA, USA). The tumor samples were denominated as diploid when only one peak was detected and aneuploid when two separate peaks were found since it was assumed that all tumor samples contained normal as well as tumor cells. Kidney cortex tissue samples were used as internal standards in each patient. The proportion of S-phase cells (%) was calculated from DNA histograms as described previously [7]. Histograms with multiple peaks, near-diploid peaks, background debris or too few cells were excluded.

PCNA evaluation with immunohistochemistry

As previously described in detail [9], all tumor samples for PCNA-IHC evaluation were routinely fixed in 10% buffered formalin and paraffin embedded. A monoclonal mouse antibody against PCNA (PC-10, Novocastria Laboratories, Newcastle upon Tyne, UK) was used. Biotin-labeled horse anti-mouse antibody, and the avidin-biotin complex method (Vectastain, Vectorlab, CA, USA) with aminoethyl-carbazole as substrate, was further used for visualization. The PCNA-IHC labeling index, i.e., the percentage of positively stained nuclei out of the total number of tumor nuclei, was calculated and about 900–1200 cells were counted in each slide.

In vivo iododeoxyuridine labeling

The patients were given an intravenous infusion over 8–12 min of 100 mg IdUrd in 100 ml saline, 3–6 h before nephrectomy. Informed consent was obtained from each patient. Samples fixed in 10% buffered formalin and paraffin wax embedded were used for IdUrd-IHCA analysis as described previously [8]. A monoclonal mouse antibody reactive with IdUrd/BrdUrd (Clone B44, Becton-Dickinson, Sunnyvale, CA, USA) was used. Methods for visualization and counterstaining were used as for PCNA-IHC. The IdUrd-IHC labeling index (%) was calculated from each slide as described for PCNA-IHC. The FCM-IdUrd method has previously been described in detail [12]. Briefly, the ethanol-fixed tumor samples were minced into fragments and nuclei were extracted. A mouse monoclonal anti-BrdUdr/IdUrd antibody (Clone Bu20a, Dako Ltd., High Wycombe, UK) was used. The samples were stained with propidium iodide solution, filtered and run in an Ortho Systems 50-H Cytofluorograph. The IdUrd-FCM labeling index (%) was calculated as described by Wilson et al. [19].

Statistical analysis

For statistical analysis the Mann-Whitney U-test was used. The differences of the values of the methods used were plotted against their means according to Bland and Altman [1].

Results

Individual parameters

Data for analysis were obtained in 136 tumor samples from 61 patients. The proliferation data from the respective parameters are summarized in Table 1. The S-FCM percentage varied from 1.0% to 18.0%, with a mean of 5.9%, which was slightly higher than for PCNA-IHC, which had a mean labeling index of 4.7%, with a range of 0.7%–18.0% ($P = 0.54$). The IdUrd methods gave significantly lower labeling indices than the S-FCM and PCNA-IHC methods ($P < 0.001$, respectively). The IdUrd-IHC labeling index ranged from 0.1% to 2.7%, with a mean of 1.2%, and mean IdUrd-FCM labeling was 1.7%, ranging from 0.2% to 8.6%.

Comparative analysis

When the various parameters were compared according to the Bland and Altman method, nearly all values were within the limits of agreement, defined as the mean difference ± 2 SD of the differences. Hence, the results showed good agreement between the methods. The data indicate that the parameters are measuring different but related aspects of cellular proliferation due to systematic differences as a function of the mean value on most comparisons. The results are graphically illustrated in Fig. 1a–f.

DNA ploidy

The data from the four proliferation methods and their relationship to DNA ploidy are summarized in Table 2. For all methods evaluated a significant difference in labeling index between diploid and nondiploid tumor samples was found ($P < 0.001$). Furthermore, the quotient between diploid and aneuploid samples was similar for the four methods (range 0.34–0.44).

Table 1 Proportion of proliferating cells evaluated by four different methods in renal cell carcinoma

	No.	Mean ± SD (%)	Median (%)	Range (%)
S-FCM	130	5.9 ± 4.4	5.2	1.0–18.0
PCNA-IHC	118	4.7 ± 2.9	4.3	0.7–18.0
IdUrd-IHC	78	1.2 ± 0.7	0.9	0.1–2.7
IdUrd-FCM	69	1.7 ± 1.4	1.4	0.2–8.6

Discussion

The fraction of proliferating cells is one important factor for tumor growth, and has been found to be a major prognostic parameter for many tumors. Our aim was to study cell proliferation parameters and DNA ploidy as supplements to the conventional prognostic variables in renal cell carcinoma [7–9, 12]. An ideal tumor cell proliferation parameter should be simple to determine, reproducible and its interpretation straightforward. Do different techniques provide comparable information on proliferation and does one methodology have any advantage over the others? Tumor heterogeneity is one factor that makes the interpretation of cell kinetic data difficult. We have previously found considerable tumor heterogeneity in renal cell carcinoma concerning S-FCM, PCNA-IHC, IdUrd-FCM and DNA content [7, 8, 11, 12]. Jain et al.

Fig. 1a–f Differences in proliferation indices versus the means according to Bland and Altman. *Dotted areas* represent 95% confidence intervals. **a** S-FCM and PCNA-IHC in 114 tumor samples. **b** S-FCM and IdUrd-IHC in 73 tumor samples. **c** S-FCM and IdUrd-FCM in 65 tumor samples. **d** PCNA-IHC and IdUrd-IHC in 60 tumor samples. **e** PCNA-IHC and IdUrd-FCM in 55 tumor samples. **f** IdUrd-FCM and IdUrd-IHC in 63 tumor samples

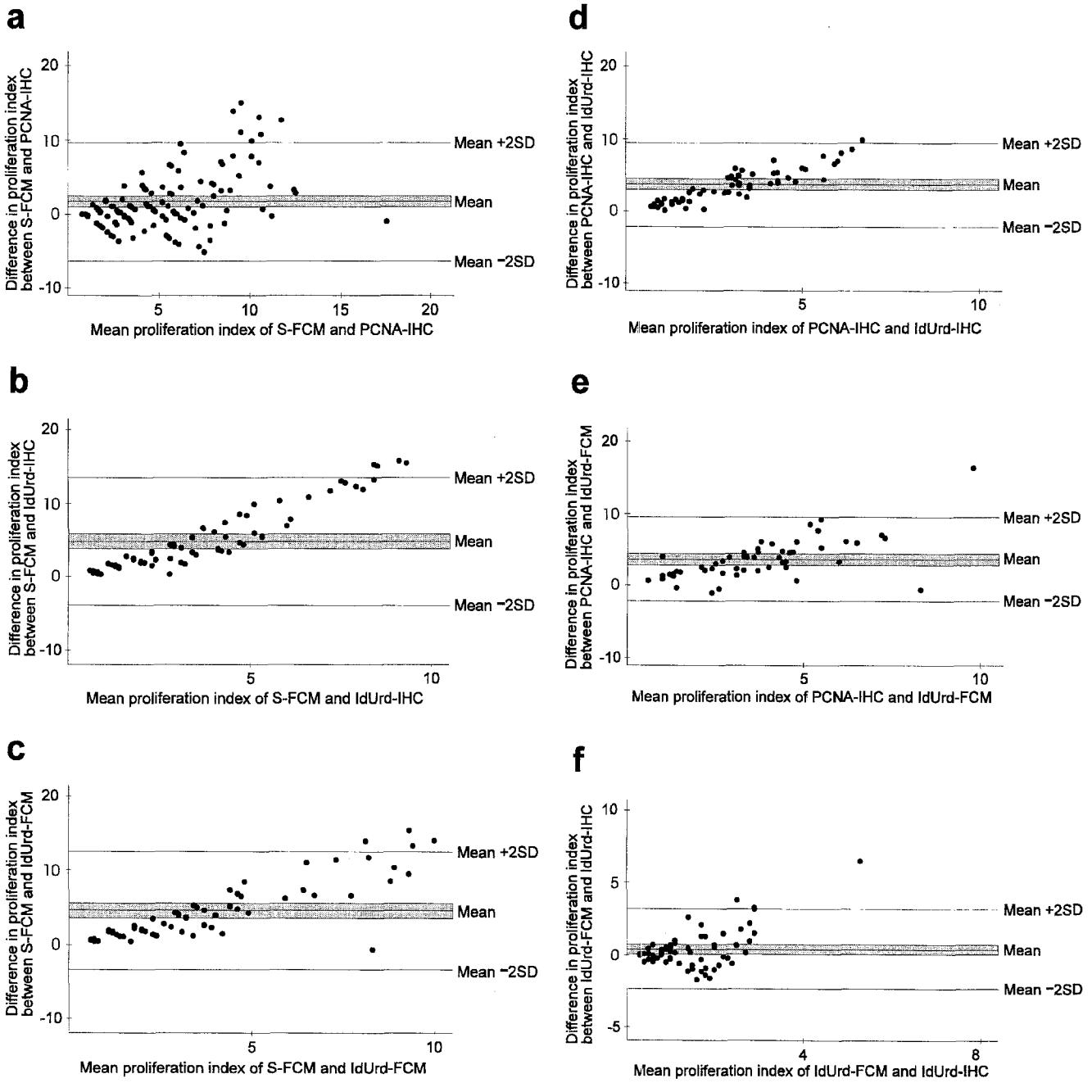

Table 2 Percentage of cells in proliferation in relation to DNA-ploidy evaluated by four methods in renal cell carcinoma

	Ploidy	No.	Mean ± SD	
S-FCM	Diploid	66	3.0 ± 1.9	$P < 0.001$
	Aneuploid	64	8.8 ± 4.2	
PCNA-IHC	Diploid	53	2.8 ± 1.5	$P < 0.001$
	Aneuploid	65	6.3 ± 2.8	
IdUrd-IHC	Diploid	35	0.6 ± 0.3	$P < 0.001$
	Aneuploid	43	1.6 ± 0.7	
IdUrd-FCM	Diploid	27	0.9 ± 0.7	$P < 0.001$
	Aneuploid	42	2.7 ± 1.5	

[5] found significant intratumoral variation in PCNA staining in a series of gastric carcinomas, and Langer et al. [6] observed a high variability of in vitro BrdUrd incorporation in renal cell carcinomas. Tumor heterogeneity warrants the examination of several representative samples from a tumor. In the present comparative study only data obtained from the same sample were accepted for evaluation, in order to minimize the influence of heterogeneity.

Our data showed that the four different techniques correlated well with each other. One advantage of the S-FCM method is that a large number of cells can easily be measured in a short time and with high accuracy, but the main drawback is that the S-phase fraction is a calculated value based on a histogram area. The method used to determine the S-phase fraction in the present study thus seemed to overestimate the proportion of proliferating cells. In a separate study we compared the model used to determine the S-phase with the RFIT model (Becton-Dickinson) and found that the two models gave very similar S-phase values (unpublished data). The DNA histogram results partly reflect the inability of this technique to discriminate between actively proliferating cells and resting or dead cells with S-phase DNA content, as well as the problem with artifacts associated with cell debris and overlapping populations. Ideally, IdUrd-IHC and IdUrd-FCM labeling indices should be at the same level as S-FCM values, since these parameters are all supposed to determine the fraction of cells in S-phase. However, the S-FCM values were significantly higher. This discrepancy indicates that the parameters do not define the same fraction of cells. The Bland and Altman comparison demonstrated that in cell populations with high proliferative activity the S-FCM technique seemed to overestimate the S-phase fraction. IdUrd incorporation in vivo is a theoretically attractive method since it monitors DNA-synthesizing cells via a well-defined pathway. The labeling efficiency will be influenced by variations in vascular perfusion restricting the delivery to tumor cells in the DNA synthesis phase. IdUrd labeling was analyzed using both IHC and FCM techniques, and somewhat higher mean labeling indices were obtained from the FCM analysis, a finding in agreement with a series on head and neck carcinomas reported by Nylander et al. [14]. For IHC evaluation there is a range in staining intensity, and only tumor cells positive beyond doubt were counted. The discrepancy may also be due to the low labeling index and because an insufficient number of cells might have been counted. When performing IdUrd-FCM analysis, the gating procedure is of importance, and inappropriate gates may include nonproliferative cell populations with an overestimation of the labeling index as a result. The main disadvantage with IdUrd labeling is the need for in vivo administration of a potentially toxic substance, which restricts its use for routine clinical practice.

The PCNA protein is present in all cycling cells with the highest expression in S-phase, and the PC-10 antibody should detect all cells in their active cycle after formalin fixation, as proposed by Garcia et al. [3]. Consequently, the PCNA labeling index is expected to be 3–5 times higher than the S-phase value, which was also found when compared with the IdUrd data. In a series of gastrointestinal lymphomas, Woods et al. [20] demonstrated similar values for the PCNA-IHC index and S-FCM analysis, but in the present study the PCNA-IHC index was actually less than the S-FCM level. This deviation might also be due to the fixation method and further indicates that the FCM techniques might overestimate the number of cells in proliferation [18]. One advantage with the IHC methods is that slide-based evaluation allows the observer to simultaneously evaluate incorporated tumor cells and histologic appearance [20]. The main drawbacks with the IHC methods are that they are relatively time-consuming procedures and the common intra- and interobserver variations.

Although there were differences in proliferation indices between the techniques, the four methods correlated well with each other. By using Bland-Altman graphics a good degree of agreement for all the compared methods was confirmed, and the best conformity was demonstrated between the IdUrd methods and between S-FCM and PCNA-IHC. When the IdUrd methods were compared with S-FCM and PCNA-IHC, skewed "tails" were obtained, suggesting that there is a systematic difference between the proliferation parameters and that they measure different but related parameters as discussed earlier. In this study there was a significant difference between diploid and aneuploid tumor samples, the former showing significantly lower labeling indices as evaluated by the four different techniques. Our findings are in agreement with Sahni et al. [15], who demonstrated significantly lower proliferation indices in diploid tumors compared with aneuploid tumors studied with in vitro BrdUrd labeling and S-FCM analysis in a series of ovarian carcinomas. In contrast to our data, Tachibana et al. [17] found no differences between diploid and aneuploid renal cell carcinomas incubated in vitro with BrdUrd.

Since the results obtained of the different techniques were related to each other, although different cell cycle compartments were monitored, the study shows that each technique can be used for monitoring cell proliferation in renal cell carcinoma. S-FCM seems to be the best proliferative parameter to be used in the clinical situation. S-FCM is relatively simple to determine, is reproducible and can be performed with a comparatively low expense. The other cell kinetic methods studied, especially the IdUrd techniques, can be recommended for further clinical studies.

Conclusion

Our study demonstrates that the four different proliferative techniques showed good agreement, and as markers for cell proliferation the methods were interchangeable. Although each technique has its limitations, assessment of cell proliferation has many potential applications and more extensive research is needed to evaluate the various techniques in the clinical setting.

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