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Proliferation Kinetics and Prognosis of Renal Cell Carcinoma

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Summary and Key Words

The historical development of different techniques for the study of proliferative patterns of normal and malignant tissue is explained. For more than 100 years, pathologists and clinicians have attempted to clarify the prognostic impact of this tumor-biological parameter. One relevant result of these investigations is the 'Grading-System', a histomorphological description of tumor cell differentiation. The autoradiographic method using tritiated thymidine (thymidine incorporation assay) detects a portion of DNA-synthesizing cells directly. However, radioactivity and long autoradiographic exposure times are the main disadvantages. DNA cytophotometry and immunohistochemical labeling are currently the most frequently used techniques for direct assessment of proliferating cells in normal and malignant renal tissue as well as in other human tumor tissues. Concerning renal cell carcinoma (RCC), our own experience and the results of the literature are compared and discussed: In contrast to tumor staging, tumor grading correlates with the tumor-specific proliferation rates. According to the present data, the measurement of the proliferative kinetics seems to be a relevant prognostic parameter for detection of high-risk patients, especially in the early stages showing identical histological findings for staging and grading.

Renal cell carcinoma · Proliferation kinetics · Autoradiographic technique · DNA-cytometry · Immunostaining

Zusammenfassung und Schlüsselwörter

Die Entwicklung der verschiedenen Techniken zur Erfassung des proliferativen Verhaltens von Normal- und Tumorgewebe beim Nierenzell-Karzinom wird beschrieben. Seit mehr als 100 Jahren beschäftigen sich Pathologen und Kliniker mit der prognostischen Relevanz dieses tumor-biologischen Parameters. Ein Ergebnis dieser Forschung ist das «Grading-System», eine histomorphologische Beschreibung der Tumorzell-Differenzierung. Die autoradiographische Methode unter Verwendung von radiomarkiertem Thymidin (Thymidineinbaurate) erfaßt direkt die Rate der DNA-synthetisierenden Zellen. Radioaktivität und lange Expositionszeiten sind die Hauptnachteile dieser Methode. Die DNA-Zytophotometrie sowie immunhistochemische Markierungsmethoden sind gegenwärtig die am häufigsten angewandten Methoden zur direkten Bestimmung des Anteils proliferierender Zellen sowohl im Normal- als auch im Tumorgewebe der Niere sowie auch anderer Tumoren des Menschen. Die eigenen Erfahrungen sowie die Ergebnisse verschiedener Autoren werden verglichen und diskutiert: Im Gegensatz zum Tumor-Staging zeigt das Tumor-Grading eine Korrelation gegenüber den tumor-spezifischen Proliferationsraten. Gemäß den Daten aus der Literatur erscheint die Bestimmung der tumor-spezifischen Proliferationskinetik als ein relevanter prognostischer Faktor zur Erfassung von Hoch-Risiko-Patienten, insbesondere in Frühstadien mit identischen histologischen Befunden im Staging und Grading

Nierenzellkarzinom · Proliferationskinetik · Autoradiographische Technik · DNS-Zytometrie · Immunhistochemie

Historical Survey

One of the main characteristics of cancer is the haphazard proliferation of malignant cells. The fraction of proliferating cells strongly influences tumor growth and is believed to be a major parameter for prognosis and treatment selection [1, 2]. For more than 100 years pathologists and clinicians have been engaged in examining proliferative patterns and behavior of human malignancies, comparing these results with normal tissue findings. This is necessary for a better understanding of pathogenesis, therapeutic principles and individual prognosis. Flammig (1882) and Waldeyer (1888) were the first to describe the histomorphological conception of 'cell division, mitosis and cell proliferation' in human malignancies [3]. In different malignant tumors, pathologists found a correlation between the mitosis rate determined in histological specimens and individual clinical course of patients with neoplasms [2, 4]. Mitosis, however, represents only a short phase in the active

cell cycle. Microscopically, only 1% – 5% of all DNA-synthesizing cells are detectable in histological specimens [5, 6, 7]. Therefore, this can only be regarded as an indirect parameter of the proliferative pattern of the tissue.

It took a long time before a technique for the direct detection of tumor specific proliferation rates was made available. By autoradiographic studies using tritiated thymidine ($[^3\text{H}]\text{-Thd}$) (=thymidine incorporation assay), direct measurement of the portion of DNA-synthesizing cells was achieved for the first time. In 1979, Rabes et al. [5] presented a clinical report of proliferation rates in renal cell carcinoma (RCC) using this technique. The authors found low proliferation rates (<20%) and a correlation to the recurrence rate in a series of RCC patients. The main disadvantages of this method, however, involve the extracorporeal organ perfusion (ex vivo) required after tumor nephrectomy, the radioactive contamination and waste disposal as well as personal safety, long autoradiographic exposure times and, thus, the lack of practicability of this technique in routine diagnosis [6, 8]. The successful immuno-

logical preparation of monoclonal antibodies represented a relevant advance in this field [9].

DNA-Cytometry for RCC

Single-cell and flow DNA-cyto(photo)metry (e.g. combined with the Feulgen staining) are commonly used techniques for the evaluation DNA-content of malignant of tissue tumors [2]. Formalin-fixed and paraffin-embedded tissue [10] as well as fresh tissue can be analyzed for DNA-cytophotometry [2]. This technique has been used for more than ten years in basic and clinical research. Extensive experience has been achieved by means of this technique: In 1985, Ljungberg was one of the first authors using DNA flow-cytometry on RCC [11]. In 196 analysed tissue samples of 25 cases of RCC, a correlation between DNA distribution (ploidy) and morphologic grading was demonstrated. Tumor proliferation, as determined by the fraction of cells in S-phase, was significantly higher in aneuploid samples compared to normal kidney tissue samples and diploid tumor samples. This paper was the first to describe DNA heterogeneity in RCC caused by different tumor cell clones. To exclude a so-called 'sampling error' several biopsies depending on the tumor size must be taken from each tumor [11]. About 40% of the analyzed RCC were aneuploid with a DNA content $> 5 C$. Different studies confirmed the prognostic importance of this parameter in RCC [12, 13].

The results of both techniques, single-cell and flow DNA-cytometry, showed no relevant differences in ploidy heterogeneity and prognostic value of DNA content for RCC patients [14].

The main disadvantage is that this technology requires equipment for photometric imaging. Due to the expense this equipment is only available in research and medical centers. Although it is a valid and reliable parameter in RCC, DNA-cytometry did not prove successful in routine diagnostics [15].

Immunohistochemical Techniques

Bromodeoxyuridine (BrdU) Technique

In order to avoid the problems of the thymidine incorporation assay, several investigators used 5-bromo-2'-deoxyuridine (BrdU), a non-radioactive thymidine analogue which is also incorporated into DNA-synthesizing cells [8, 16, 17, 18, 19]. In 1982, Gratzner et al. isolated and described a monoclonal antibody (mab) against BrdU [20] which allows for the rapid detection of BrdU-laden cells in vivo and in vitro as well as by immunohistochemical techniques [21]. Although BrdU immunohistochemistry solves many of the problems associated with [3H]-Thd in cell kinetics studies (as outlined above), its use generates the following problems: BrdU is a cytotoxic substance. Administrations of BrdU at high doses have been shown to be mutagenic in vitro and carcinogenic in vivo in an animal model [22]. BrdU also inhibits cell differentiation and thymidine uptake in vitro, as well as DNA synthesis and cell replication in both in vitro and in vivo settings [8]. Whether or not these negative aspects of BrdU administration affect the results compiled by in-vivo cell kinetic studies, remains un-

clear. Due to the mutagenic and carcinogenic potentials, BrdU can only be used for animal and in-vitro studies, but not for direct in-vivo determination of proliferative cell fractions in human malignancies. Therefore, the BrdU immunohistochemistry represents an indirect method only to assess the proliferation rates in human neoplasms.

Ki-67 Staining

In 1983, Gerdes et al. isolated and characterized a human nuclear antigen (termed 'Ki-67') present in DNA-synthesizing cells only [23]; its expression is observed in normal as well as in malignant cells but is absent in resting cells [24]. Figure 1 illustrates this schematically for the different phases of cell division [24]. Using frozen sections and immunohistochemical staining techniques as outlined by Gerdes [25] and Cordell [26], individual in-vivo proliferation rates can now be obtained directly in normal and malignant tissue as well (fig. 2).

Ki-67 is a mouse monoclonal antibody which binds to a human nuclear antigen associated with cell proliferation [23, 24]. Cell cycle analyses demonstrated that the nuclear antigen detected by Ki-67 is only expressed in the G_1 , S, G_2 and M phases of proliferating cells, normal as well as malignant, but is absent in resting cells (G_0) (fig. 1) [24, 25]. Immunostainings with Ki-67 revealed nuclear reactivity in cells of germinal centres of cortical follicles, cortical thymocytes, neck cells of gastrointestinal mucosa, undifferentiated spermatogonia and cells of a number of human cell lines. The Ki-67 antibody did not react with cells known to be in a resting stage such as lymphocytes, monocytes, parietal cells and Paneth's cells of gastrointestinal mucosa, hepatocytes, normal renal cells, mature sperm cells and brain cells [23]. Expression of this antigen recognized by Ki-67 could be induced in peripheral blood lymphocytes after

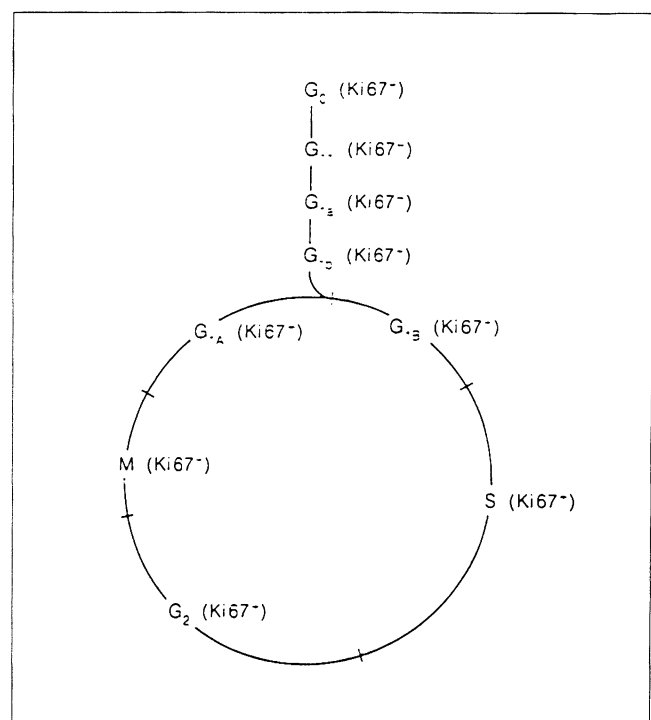


Fig. 1. Occurrence of cell proliferation-associated nuclear antigen (Ki-67) in the different phases of cell cycle [24]. + = antigen expression; - = no antigen expression.

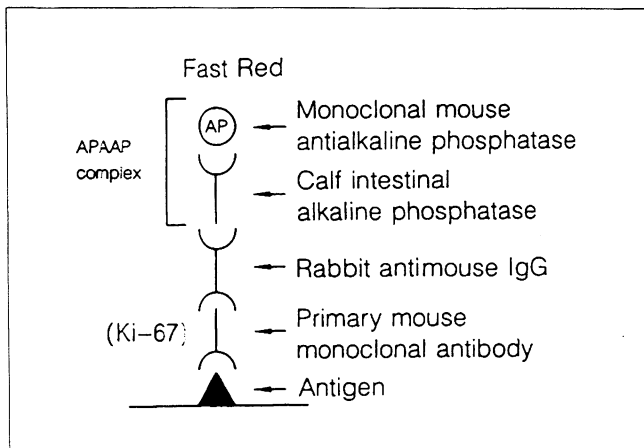


Fig. 2. Immunocytochemical labeling of a proliferation-associated nuclear antigen using the mab Ki-67 and the alkaline phosphatase anti-alkaline phosphatase (APAAP) complex: Positive cells are stained red, hemalum is often used for counterstaining (\rightarrow blue).

stimulation with phytohaemagglutinin, whereas it disappeared from HL-60 cells stimulated with phorbol esters to differentiate into mature macrophages in the resting stage [5]. In the meantime, numerous reports of immunohistochemical investigations on normal and malignant human tissues have confirmed the Ki-67 staining assay to be an easy, practicable, reliable and reproducible method for evaluating the proportion of proliferating cells (proliferation rates) [2, 27, 28, 29, 30, 31].

Ki-67 staining is performed as follows: Tissue biopsies must be stored in fluid nitrogen to get 5 μ frozen sections for the immunohistochemical staining; formalin fixed samples are not suitable [25]. As recently described, growth fractions of cultured tumor cells can also be immunocytochemically determined on special slide chambers [15, 31].

Ki-67 staining is delineated schematically in figure 2: The acetone- and chloroform-fixed cells (in vivo: 5 μ frozen sections; in vitro: cells are directly attached on the slides [15]) are exposed for approximately 30 minutes to the monoclonal antibody (Ki-67) which has been diluted in RPMI 1640 medium. After washing in TRIS-buffered solution (pH 7.4–7.6), the cells are incubated with rabbit-anti-mouse IgG and then exposed to APAAP (alkaline phosphatase anti-alkaline phosphatase) complex after repeated washing [26]. Subsequently, the slides are stained with 'Fast Red' and counterstained with hemalum [25, 31]. Presence of the specific proliferation-associated nuclear antigen is indicated by a red instead of a blue color reaction after counterstaining (fig. 3, 4).

Different human malignancies have already been investigated using the Ki-67 immunohistochemistry [1, 2, 15, 27, 29, 30, 32, 33, 34, 35]: The in vivo proliferation rate of RCC is low, only ranging up to 25% [15, 36]. Because of this low proliferative potential, only a few mitotic cells can be obtained by biopsy. For that reason, direct karyotyping of the cells is impaired by irregular contractions of the chromosomes [37, 38, 39]. Therefore, reports on the chromosomal features of RCC are rare. Yoshida et al [39] were among the first to karyotype malignant renal cells after short-term in vitro culturing.

During short-term in vitro culturing, the proliferation rates of

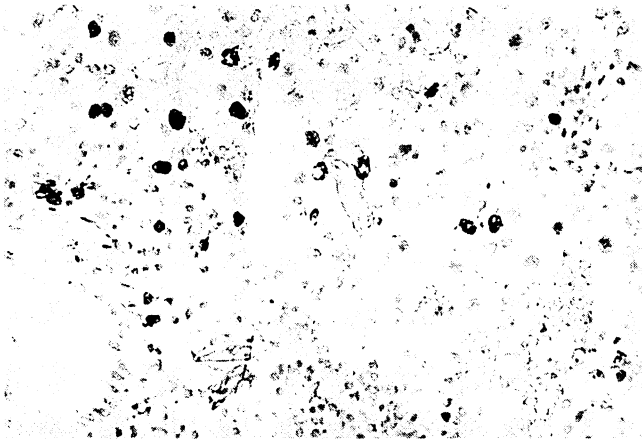


Fig. 3. Immunohistochemical in vivo staining of an undifferentiated renal cell carcinoma using the Ki-67 technique. Positive cells are stained darkly. Proliferation rate (PR) = 15%: original magnification $\times 120$.

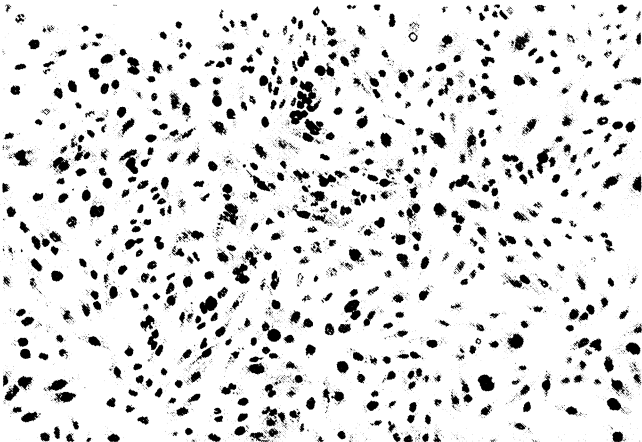


Fig. 4. In vitro short term cell culture of the same renal cell carcinoma as demonstrated in figure 3, immunocytochemical staining obtained using mab Ki-67, positive cells are stained darkly. PR = 82%: original magnification $\times 120$.

malignant renal cells increase up to 85%. This phenomenon improves the cytogenetical output [15]. Malignant renal cells prepared in vitro in the manner as recently described were characterized by employing conventional cytological, cytochemical and immunocytochemical measures [40]: Malignant renal cells were exclusively obtained by the cell-cluster preparation technique [7]. Aberrations on the short (p) arm of chromosome 3 were the most common cytogenetic finding (77.8%) in 36 cases of non-familial RCC. It seems that this chromosomal defect is the initial cytogenetic change and that it triggers genetic instability during the clonal development and progression of human RCC [7, 15]. These connected findings could be appropriately verified by the described Ki-67 immunostaining assay [15].

There are different methods to determine the percentage of Ki-67-positive cells; one is described by Gerdes [25]. In recent years, the immunostaining quantitative analysis has been done by means of a computer-assisted image processor configured with a standard microscope [27]. The highest proliferation rate found for all specimens in a given case is considered to be

representative of the entire tumor. To exclude sampling errors in the determination of the tumor-specific proliferation rate, several samples of solid tumor tissue must be obtained, depending on the individual tumor size (table 1) [15]. This experience is also described in other malignant soft tissue tumors, taking into account that several biopsies are necessary to obtain the intratumoral variability [27].

As illustrated in figure 5, fresh tumor tissue must be stored immediately in fluid nitrogen for correct and reliable determination of the tumor-specific proliferation rate [15]. Rates measured 3.5 h after tumor nephrectomy amounted to only approximately 50% of those initially determined [15]. This observation correlates with in vitro growth rates of tumor cells, which depend essentially on the interval between nephrectomy, tissue sampling and fresh tissue preparation [40, 41].

Tumor infiltrating lymphocytes often observed in the peripheral zones of RCC should be excluded in the immunohistochemical Ki-67 evaluation. These lymphocytes exhibit cell proliferation within the tumor which are obviously caused by lymphokines and can therefore simulate higher proliferation rates of the tumor. For this reason, reference slides of each biopsy must be taken for conventional histological examination to exclude this phenomenon on frozen sections; histological differentiation between lymphocytes and tumor cells is almost impossible on frozen sections alone [15].

Confirming the data published by Rabes et al. [5, 6], higher proliferation rates were always measured in the peripheral zones of the tumors as compared with central zones [15]. This is obviously nutrition-related due to better blood circulation in the peripheral areas. According to these findings, it is obvious that necroses and cysts initially develop in the central zones of renal tumors, a phenomenon often observed macroscopically [42].

Urological and other solid human malignancies exhibited similar proliferation rates (defined by the mab Ki-67) to those already mentioned for RCC [15, 36]:

Transitional cell carcinomas of the bladder:

Histological grade 1 tumors showed proliferation rates ranging between 1%–5%, whereas grade 3 tumors yielded up to 30% [1].

Prostatic carcinoma:

Low-grade tumors: 1%–3%. High-grade tumors: 10%–18% [32].

Non-small cell lung cancer:

Low-grade tumors: 1%–5%. High-grade tumors: 15%–20% [27].

Table 1. Number of specimens according to tumor size for representative measurement of PRs

	Peripheral (n)	central (n)
< 3 cm	2	1
3–6 cm	4	2
> 6 cm	6	2
	PR	
	peripheral > central	

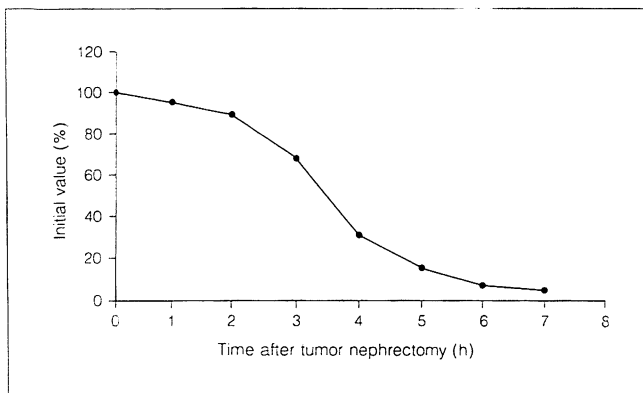


Fig. 5. In vivo proliferation rates plotted against the time of specimen collection (mean values of ten different tumors).

Breast cancer:

Low-grade tumors: 2%–12%. High-grade tumors: 13%–20% [28, 34, 35].

Up to now, only a few papers have dealt with the tumor-specific Ki-67 determination in RCC [15, 36, 43, 44], revealing a correlation between proliferation rates and histological grading, but almost no correlation with tumor stage.

The individual tumor stage cannot be concluded from the proliferation status, as it is a function of cell proliferation as well as of the time of tumor growth, the latter parameter not being measurable at the time of diagnosis. Some large RCC exhibited low proliferation rates, where as low-stage tumors (pT₁₋₂: 2–4cm in diameter) were found to vary 5-fold in proliferation rates [15]. These data explain the clinical observation in cases of RCC in which, despite a non-surgical approach for various reasons, an almost proliferative arrest was noted at long-term follow-up [45]. On the other side unusual growth rates of low-stage were observed by imaging techniques (CT, sonography) [46, 47]. A similar correlation is found in other malignancies using the Ki-67 immunohistochemistry revealing a correlation between proliferation status and tumor grading, but no correlation with tumor stage [2, 28, 34, 35].

Only a few authors have outlined the correlation of proliferative behavior of different malignancies and prognosis. Measurement of the individual proliferation status appears to be an additional diagnostic parameter for the detection of RCC patients at high risk, especially in the early stages showing identical histological features [15, 36, 43, 44].

In breast cancer, the immunohistochemical Ki-67 parameter was found to be an additional independent prognostic parameter beyond conventional histological staging and grading [28, 34]. Further investigations are necessary to study immunohistochemistry and long-term follow-up of patients to confirm this preliminary experience.

Comparative studies verified the equivalence of DNA cytometry and immunohistochemical Ki-67 index in assessing the proliferative cell fraction of normal and malignant tissue [2]. Because immunohistochemical staining techniques are employed in almost all pathological institutions, the Ki-67 staining assay will, in the future, most probably be used for routine histological diagnostics. Immunohistochemical determination of tumor-specific proliferation rates using the monoclonal antibody (mab) Ki-67 is a practicable, reproducible and reliable method

to obtain the proliferative pattern of RCC and other human malignancies. It may be important for the detection of high-risk patients and, conclusively, for prognosis.

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