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# Proliferative behaviour and cytogenetic changes in human renal-cell carcinoma

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**Summary.** From 1986 until 1990, in vivo proliferation rates (PRs) in 110 patients with renal-cell carcinoma (RCC) were immunohistochemically determined by the Ki-67 assay. It could be demonstrated that the PRs of RCCs range between only 1% and 15%. Due to its low proliferative kinetics in vivo, tumor cytogenetic investigations of this malignancy remain rare. During short-term in vitro culture, the PRs of this neoplasm increased (21% – 82%). Therefore, 36 untreated human RCCs were cultured in vitro for cytogenetic analysis using the G-banding technique. In all, 77.8% (28/36) of the renal malignancies investigated exhibited an aberration of chromosome 3, which seems to serve as a marker for this malignancy. Whereas tumor stage showed no correlation with PR, tumor grade exhibited a strong correlation with this parameter. According to the data presented herein, immunohistochemical determination of the tumor-specific PR using the monoclonal antibody Ki-67 is a practicable, reliable and reproducible method that complements conventional histological tumor grading and staging. This parameter appears to be useful in identifying RCC patients at high risk, especially at early stages that are identical in tumor stage and grade.

For > 100 years, pathologists and clinicians have been engaged in examining proliferative patterns and behaviour of human malignancies as compared with normal tissue. This is necessary for a better understanding of pathogenesis, therapeutic principles and individual prognosis. Flamming (1882) and Waldeyer (1888) were the first investigators to describe the histomorphological conception of "cell division, mitosis and cell proliferation" in human malignancies [8]. In different malignant tumors, pathologists have found a correlation between the mitosis rate determined in histological specimens and the individual clinical course of patients with neoplasias [10, 18]. 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 [15, 17]; therefore, this can be regarded only as an indirect parameter of the proliferative pattern of the tissue.

It took a long time for the direct detection of tumor-specific proliferation rates (PRs) to become possible. By autoradiographic studies using the thymidine incorporation assay, direct measurement of the portion of DNA-synthesizing cells was achieved for the first time. In 1979 Rabes and co-workers [15] presented a clinical report of PRs in renal-cell carcinoma (RCC) using this technique. These authors found low PRs (< 20%) and a correlation between PR and recurrence rate in a series of ten RCC patients. The main disadvantages of this method, however, involve (a) the extracorporeal organ perfusion (ex vivo) required after tumor nephrectomy, (b) the exposure of patients to radioactivity and, thus, (c) the lack of practicability of the technique in routine diagnosis.

The successful preparation of monoclonal antibodies represented a relevant advance in this area [12]. In 1983 Gerdes et al. [5] isolated and characterized a human nuclear antigen (termed Ki-67) present only in DNA-synthesizing cells; its expression is present in normal as well as in malignant cells but is absent from resting cells [7]. Using frozen sections and immunohistochemical staining techniques as outlined by Gerdes et al. [6] and Cordell et al. [4], individual in vivo PRs in normal and malignant tissue can now be obtained directly.

In recent years an increasing number of chromosomal aberrations have been described in human hematopoietic neoplasms [13, 23]. This finding emerges from the proliferative behaviour and the high mitotic rates of these malignancies in vivo. Because of the low spontaneous PR in vivo (only up to 15%), tumor cytogenetic investigations of human RCC remain rare [16, 22]. Direct in vivo preparation of malignant renal tissue obtains only a few mitotic cells; furthermore, karyotyping of the cells is impaired by irregular contractions of the chromosomes [1]. In the present study, the technique of in vitro preparation of human RCC is described, which enables the attain-

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ment of higher numbers of proliferative malignant cells and, thus, reproducible chromosomal findings. Furthermore, individual in vivo PRs are correlated with conventional tumor stage and grade as well as with the clinical courses of the corresponding RCC patients.

## Patients and methods

A total of 110 patients with RCC who showed no evidence of metastatic disease at the time of surgery ($N_0$, $M_0$ according to imaging techniques) entered this ongoing study for measurement of individual PRs in vivo. Successful short-term in vitro culture and banding analysis was successful in 36 of 47 cases (76.6%). The in vivo/in vitro PRs were compared in all 36 of these instances. Table 1 illustrates tumor staging and grading of the 36 cases of RCC analysed.

. Normal and malignant renal tissues were obtained by conventional radical nephrectomy. A macroscopically homogeneous area that was free of fibrosis, necrosis or haemorrhage of the tumor was excised for preparation. Figure 1 exhibits schematically the tumor cell preparation. Samples of excised normal and malignant renal tissue were taken for in vitro cell preparation, and other samples were obtained for histological examination as reference slides (H&E staining). Furthermore, samples were stored in fluid nitrogen for frozen sectioning followed by immunohistochemical analysis and measurements of the in vivo PRs as described elsewhere [6].

### *Preparation of primary cell cultures according to the cell-cluster technique*

After mincing and enzymatic treatment with collagenase, the fragments of fresh tissue were centrifuged and washed twice in RPMI 1640 medium. Subsequently, the tissue clusters were resuspended in a centrifuge tube containing 2–4 ml culture medium (RPMI 1640 supplemented with 15% foetal calf serum and 1% penicillin) and vigorously dispersed by means of a Pasteur pipette. The suspension was allowed to sediment for 1 min, and the supernatant containing >90% of the single cells with low viability and cell debris was decanted. The pellet, whose content comprised by >90% small clusters of approximately 10–15 cells each, was resuspended and seeded in culture. The cell clusters were incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$ in 25-$cm^2$ Falcon flasks (Biochrom, Berlin, FRG), to each of which 5 ml culture medium had been added. The small cell clusters attached to the flasks within 24 h and grew to an almost confluent monolayer, in most cases within 7–14 days of cell culture [13, 17].

### *Chromosome analysis*

Cytogenetic analysis was carried out on days 7–14 of primary culture, depending on the proliferation of the tumor cells. In all, 0.1–0.2 µg/ml colchicine (30–60 min) or 0.02 µg/ml colcemid (2–3 h) was added to the cell cultures. The cells were detached by 0.025% trypsin-ethylenediaminetetraacetic acid (EDTA) solution and then treated with 0.075 *M* potassium chloride solution for 15–30 min. After this hypotonic treatment, the cells were fixed two or three times with cold methanol-acetic acid (3:1, v/v). Chromosome preparations were obtained using the air-drying technique and the slides were kept at room temperature for 3–5 days before banding. Karyotype analysis was routinely performed using the (GTG) [20] and (CBG) techniques [21] and, in some cases, the (QFQ) technique [2] for the identification of the Y chromosome. Up to 15 metaphases were karyotyped in each case, with only well-banded metaphases being karyotyped according to the International System for Cytogenetic Nomenclature (ISCN) [11].

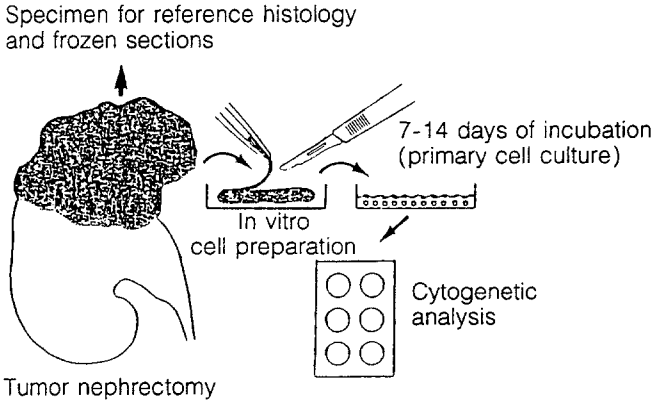


**Fig. 1.** Schematic representation of tumor-cell preparation for in vitro culturing and cytogenetic analysis

**Table 1.** Tumor stage and grade of the 36 RCCs investigated for in vivo/in vitro PRs and cultured in vitro for tumor cytogenetic analysis

| | Patients | |
|---|---|---|
| | (*n*) | (%) |
| Stage | | |
| pT 1–2 | 9 | 25 |
| pT 3 | 23 | 63.9 |
| pT 4 | 4 | 11.1 |
| Grade | | |
| G 1 | 11 | 30.6 |
| G 2 | 10 | 27.8 |
| G 3 | 15 | 41.6 |

### *Analysis of the constitutional karyotype*

To define the constitutional karyotype of the patients, normal kidney tissues were prepared for in vitro cultures in the same manner described above for tumor samples. Cytogenetic analysis was carried out by the same procedure. When normal renal tissue was not available or normal renal cells did not grow in vitro, phytohaemagglutinin-stimulated peripheral blood lymphocytes were karyotyped.

### *Preparation of in vivo and in vitro slides for determination of PRs*

As outlined above, normal and malignant renal tissues were stored in fluid nitrogen. Frozen sections were prepared for the immunohistochemical Ki-67 assay as described by Gerdes and co-workers [6] so as to obtain the in vivo PR directly. Furthermore, cells of the primary cell culture that had been transferred to cell-culture flasks were directly placed in special in vitro slide chambers (Fig. 2; NUNC Company, Wiesbaden, FRG). On the day during which the chromosomal analysis began, the in vitro slides were also stained for immunocytochemical Ki-67 assay [6, 16].

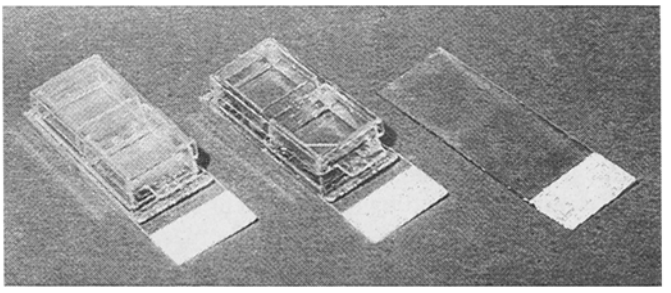

**Fig. 2.** Slide chamber for in vitro growth of malignant renal cells

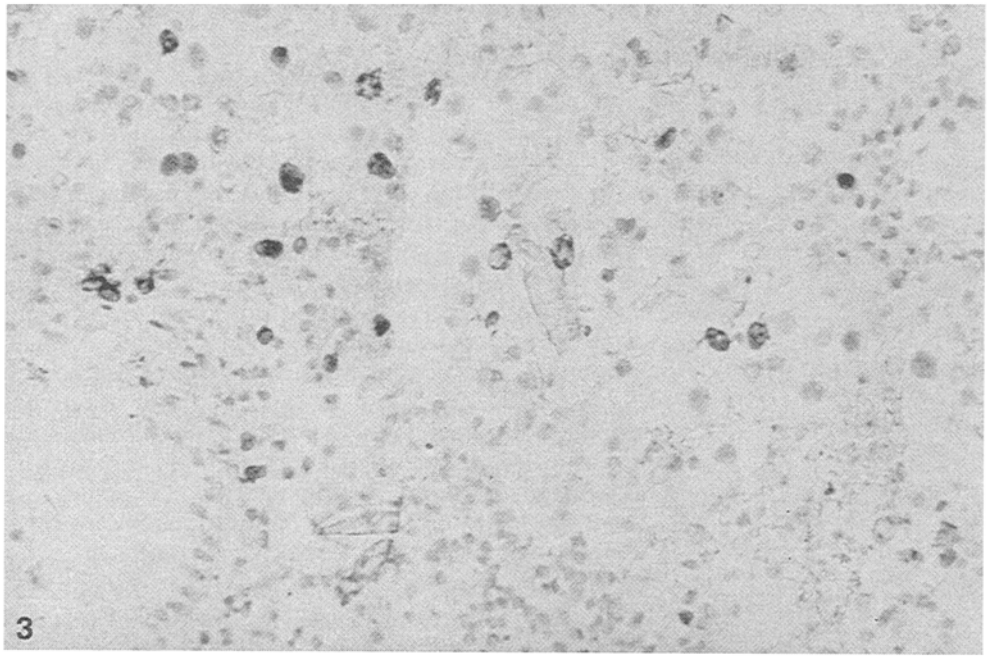


**Fig. 3.** Immunohistochemical in vivo staining of an undifferentiated RCC using the Ki-67 technique. Positive cells are stained darkly. PR, 15%; original magnification, ×120

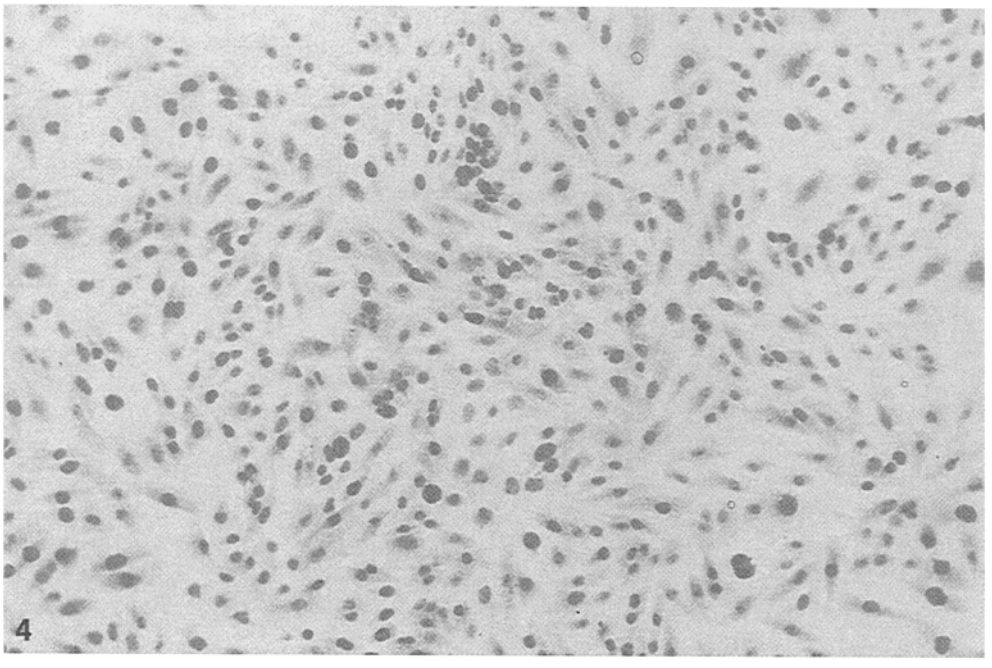


**Fig. 4.** In vitro short-term cell culture of the same RCC illustrated in Fig. 3, showing immunocytochemical staining obtained using mAb Ki-67. PR, 82%; original magnification, ×80

Gerdes and colleagues [5, 7] provided the monoclonal antibody Ki-67, which binds to a human nuclear antigen associated with cell proliferation. Cell-cycle analyses demonstrated that this nuclear antigen was detected by Ki-67 only in the $G_1$, S, $G_2$ and M phases of proliferating cells, regardless of viability, but not in $G_0$ cells [7]. Thus, the Ki-67 assay established an easy, fast and reproducible method of determining the growth fractions of tumors in vivo [6] as well as in vitro [16]. The Ki-67 staining method was performed as follows. The acetone- and chloroform-fixed cells were exposed for approximately 30 min to monoclonal antibody that had been diluted in RPMI 1640 medium. After washing in TRIS-buffered solution (pH 7.4–7.6), the cells were incubated with rabbit-anti-mouse IgG and then exposed to APAAP (alkaline phosphatase anti-alkaline phosphatase) complex after another washing [4]. Subsequently, the slides were stained with fast red and counterstained with hemalum [6, 16]. Presence of the specific antigen was indicated by a red instead of a blue color reaction after counterstaining.

## Results

Table 1 delineates the histological grade and stage of all 36 RCCs assigned to this study. As demonstrated, culturing of not only undifferentiated tumors but also low-grade RCCs was successful. The success rate for in vitro culture was 87.2% (41/47), but correct banding for tumor cytogenetic analysis succeeded in only 36 cases (76.6%). Whereas normal renal tissue showed PRs of around 1% and well-differentiated RCC tumors exhibited PRs ranging between 1% and 3%, poorly differentiated tumor tissues showed values of up to 15% (Fig. 3). Following in vitro culture, PRs of malignant renal tissues in-

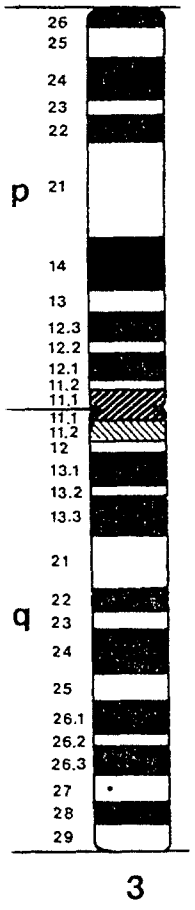


**Fig. 5.** Schematic representation of a normal chromosome 3 according to the ISCN classification [11]

creased considerably, ranging between 21% and 82% (Fig. 4).

Figure 5 gives a schematic representation of a normal chromosome 3. Figure 6 illustrates the G-banded karyotype of an in vitro cultured RCC, showing a deletion of the short (p) arm of chromosome 3, which was the chromosome most involved in tumor-induced aberrations. In 28 (77.8%) of the 36 RCCs analysed, various aberrations on chromosome 3 were observed, including deletion of the short arm or translocation of different chromosomal segments to the deletion-altered portion of the chromsome. Six tumors exhibited aberrations on chromosome 3 exclusively, showing no changes in other chromosomes (Fig. 7). In 22.2% of the cases (8/36), aberrations on chromosomes other than chromosome 3 were observed (Fig. 8). Chromosomal aberrations were noted neither in normal kidney cells nor in peripheral blood lymphocytes.

As illustrated in Fig. 9, the shortest possible interval between sampling and storage of the specimens is essential for the correct determination of individual PRs. Rates measured at 3.5 h after tumor nephrectomy amounted to only approximately 50% of those initially determined.

The determination of the percentage of Ki-67-positive cells was performed as described by Gerdes et al. [6]. To exclude sampling errors, several samples of solid tumor tissue must be obtained, depending on the individual tumor size (Table 2). The highest PR found for all speci-

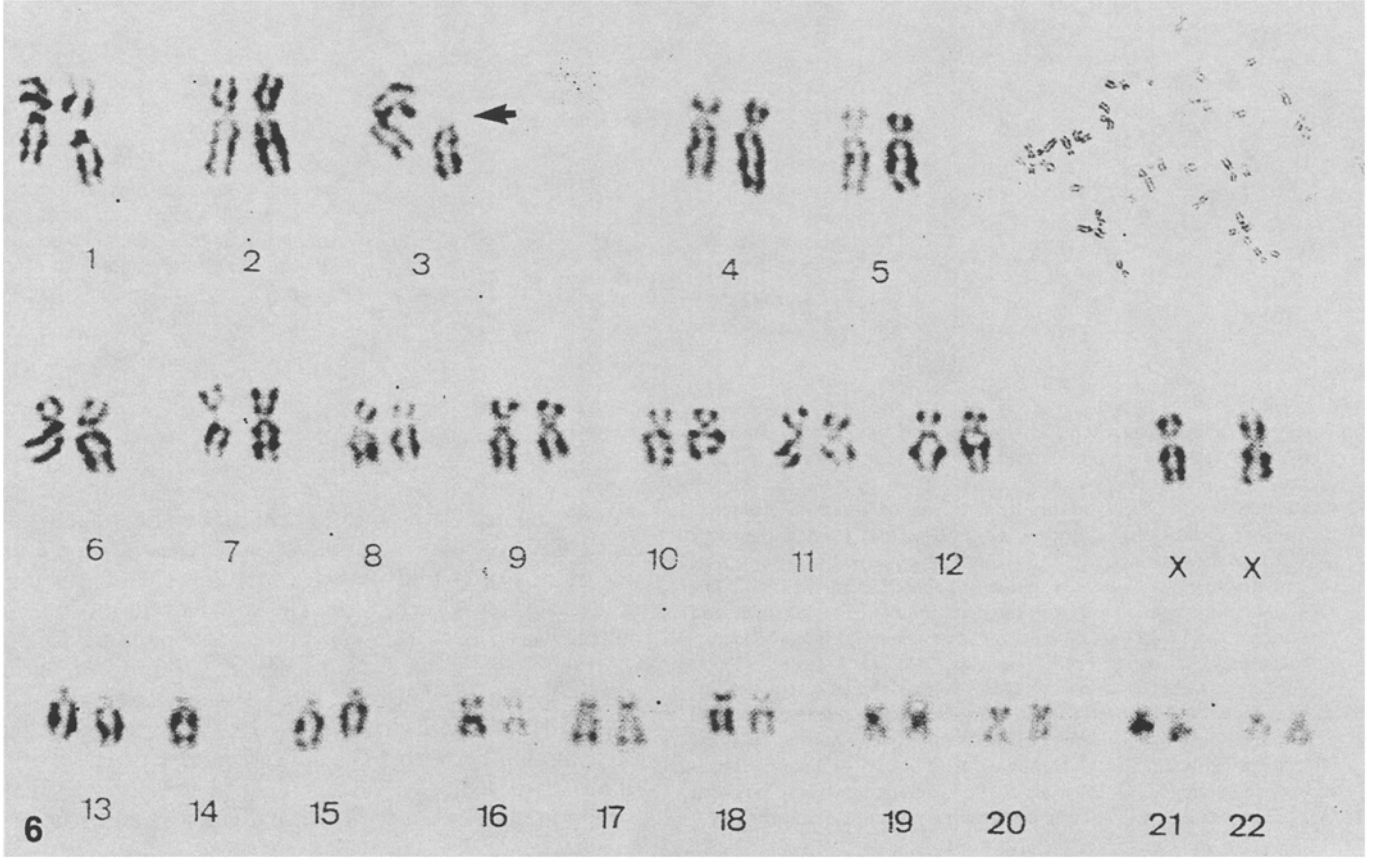


**Fig. 6.** G-banded karyotype of an RCC cultured in vitro; note the deletion of both short (p) arms of chromosome 3 (*arrow*)

**Table 2.** Number of specimens according to tumor size for representative measurement of PRs

| Tumor size | Specimens (*n*) | |
|---|---|---|
| | Peripheral | Central |
| < 3 cm | 2 | 1 |
| 3 – 6 cm | 4 | 2 |
| > 6 cm | 6 | 2 |

Proliferation rates: peripheral > central

mens analysed in a given case was considered to be representative of the entire tumor.

From 1986 until 1990, PRs in 110 patients with RCC were immunohistochemically measured using the Ki-67 assay. As determined using histology and imaging techniques, these patients showed no evidence of metastatic spread at the time of tumor nephrectomy. Table 3 gives an overview of the PR, histological tumor staging (pT) and grading (G) of the 110 cases investigated. The classifica-

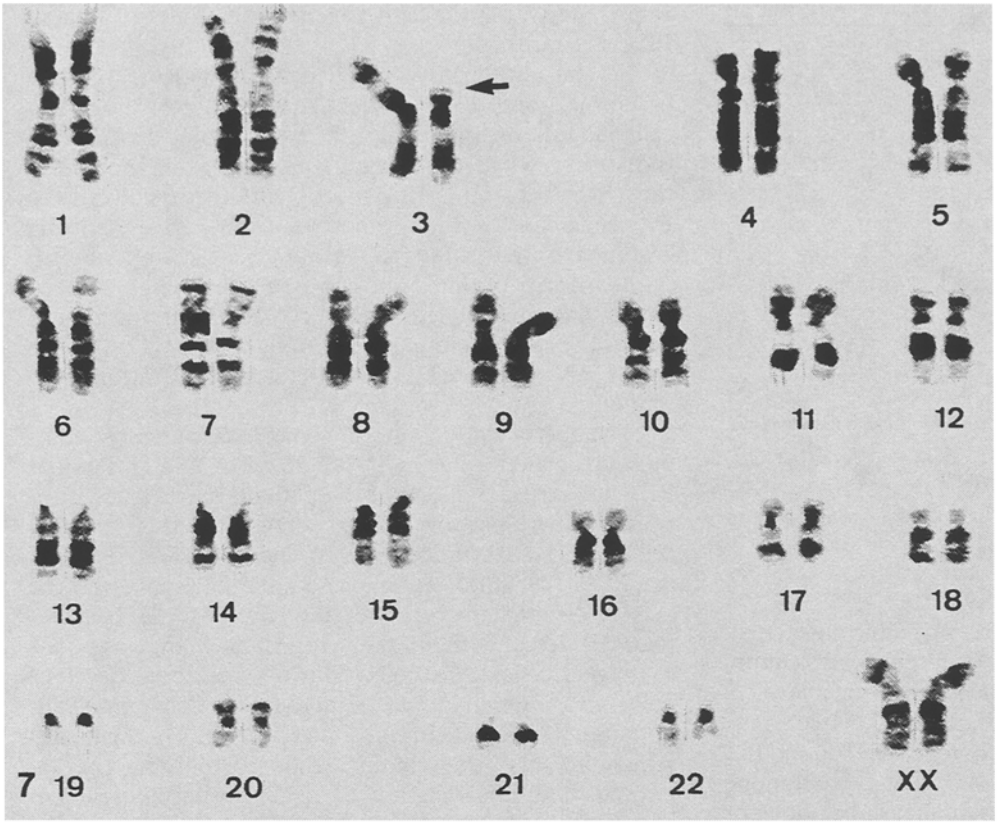


**Fig. 7.** Another case of a karyotyped RCC; deletion of one short (p) arm of chromosome 3 is the only aberration (*arrow*)

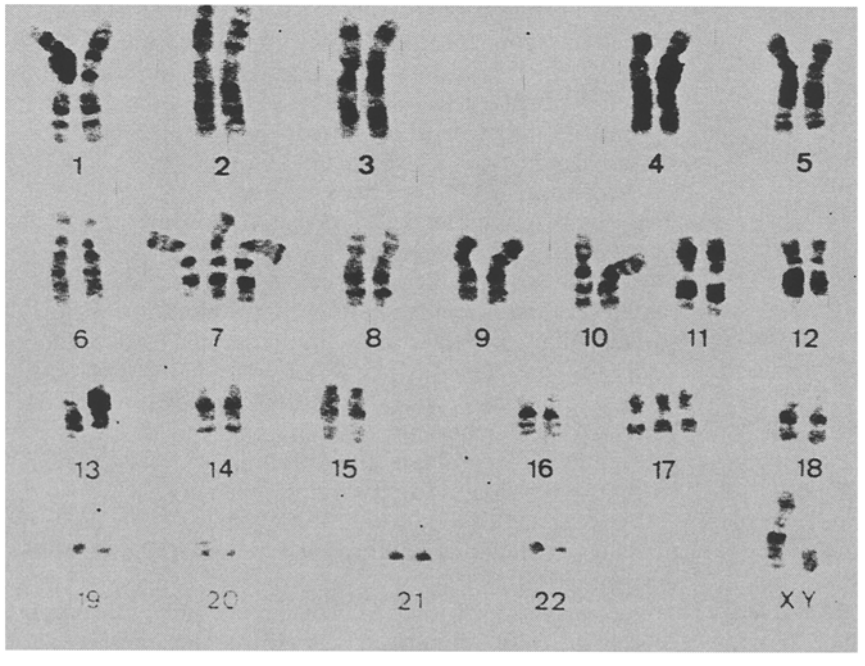


**Fig. 8.** G-banded karyotype of an RCC cultured in vitro; note the 2 normal chromosomes 3 combined with trisomy of chromosomes 7 and 17

**Table 3.** Correlation of tumor stage and grade with PRs

| | PRs ($n = 110$)[a] | | |
|---|---|---|---|
| | 0% – 4% | >4% – 9% | >9% |
| Stage | | | |
| pT 1 – 2 | 31 | 24 | 16 no |
| pT 3 | 10 | 17 | 12 correlation |
| Grade | | | |
| G 1 | 25 (74%) | 7 | 2 |
| G 2 | 24 | 12 | 14 |
| G 3 | 3 | 8 | 15 /58%) |

[a] 1986 – 1990, T1 – 3, $N_0$, $M_0$; $P < 0.001$

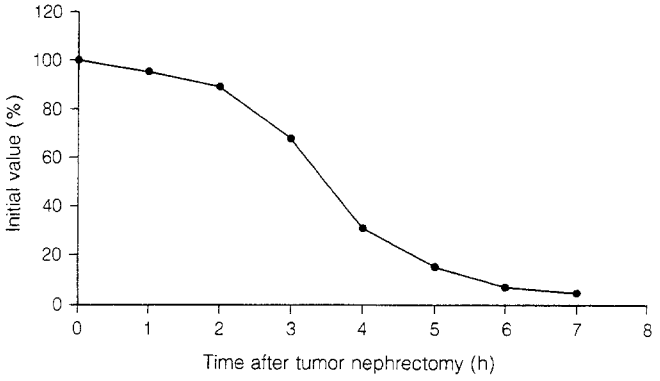


**Fig. 9.** In vivo PRs plotted against the time of specimen collection (mean values of 10 different RCC tumors)

tion of all RCC tumors was done according to the actual (TNM) system of the IUCC (International Union Against Cancer) [9]. There was no correlation between individual PR and tumor stage (pT; Table 3). In contrast to these findings, a strong correlation between PR and tumor grade was observed (G; Table 3). In low-stage tumors (pT1 – 2), 5-fold variations in PR were measured; we noted large tumors with low PRs and small tumors (< 3 cm in diameter) with PRs of >9%.

Thus far, 15 (16.3%) of the 110 patients investigated in the present ongoing study have developed relapsing tumors (mean follow-up, 2.8 years). In all, 11 cases whose tumor recurred during the follow-up period exhibited PRs of >9% at the time of tumor nephrectomy; the remaining 4 patients showed PRs ranging between 4% and 9%. Three patients with low-stage tumors (pT1 – 2) whose disease recurred showed PRs of >9%.

## Discussion

RCC occurs spontaneously and is hereditary as well. A reciprocal translocation 3; 8 was reported by Cohen et al. [3] in ten members of a family that was predisposed to RCC. These authors suggested that this chromosomal rearrangement predisposes carriers to develop RCC. Familial RCC is rare; non-familial renal cancer occurs much more frequently. However, reports on the cytogenetic features of this disease are scanty. Yoshida et al. [22] and other authors [1] have reported on several cases of cytogenetic analyses in RCC using short-term in vitro cultures. Although karyotyping was not described in detail in all of the cases, several structural changes in chromosome 3 were observed.

The in vivo PRs in RCC are low, ranging up to only 15% as determined by the immunohistochemical Ki-67 assay (see Fig. 3). Because of this low proliferative potential, only a few mitotic cells can be obtained by biopsy; furthermore, direct karyotyping of the cells is impaired by irregular contractions of the chromosomes [1, 22]. Therefore, reports on the chromosomal features of human RCC are rare.

Several authors have described different aberrations on chromosome 3 using direct karyotyping of the tumor cells [3, 13]. Yoshida et al. [22] were among the first authors to karyotype malignant renal cells after short-term in vitro culture. Following short-term in vitro culture, the PRs increase, which improves the cytogenetically evaluable output. Malignant renal cells prepared in vitro in the manner outlined above were recently characterized using conventional cytological, cytochemical and immunocytochemical measures [17]. Malignant renal cells were exclusively obtained by the cell-cluster preparation technique.

The aberration on chromosome 3 was the most common cytogenetic finding in the 36 cases of non-familial RCC investigated. None of the patients showed constitutional aberrations on chromosome 3 of their normal cells. Structural changes in chromosome 3 were detected in 77.8% (28/36) of cases; these alterations involved the loss of different segments of the chromosome. It seems that this defect is the initial cytogenetic change and that it triggers genetic instability during the clonal development and progression of human RCC. Other non-random and accidental chromosomal changes represent subsequent events. Our findings thus corroborate the hypothesis that the deletion of a chromosomal segment coding for suppression results in uncontrolled proliferation of normal tubular cells. The duration of this phenomenon accounts for malignant transformation in cases of RCC. These chromosomal aberrations were not observed in normal cells of the RCC patients; therefore, the 3p deletion must be an acquired, specific chromosomal defect in non-familial RCC.

According to the data presented herein, chromosome 3 can be described as the marker chromosome of RCC. Becasue the loss of segments on the short arm of chromosome 3 was not microscopically demonstrable in 8 of the 36 tumors, it seems possible that a mutation or loss of DNA may take place at a submicroscopic level, analogous to that demonstrated for retinoblastoma and Wilms' tumor as previously outlined by Kovacs and coworkers [13]. Further molecular biology studies, however, are required to evaluate the meaning of this specific chromosomal defect for the development of RCC.

As outlined in Fig. 9, fresh tumor tissue must be immediately stored in fluid nitrogen for correct and reliable determination of the tumor-specific PRs. This observation correlates with the in vitro growth rates of tumor cells, which also essentially depend on the interval be-

tween nephrectomy and tissue sampling [17]. Confirming the data published by Rabes et al. [15], higher PRs were always measured in the peripheral zones of the tumors as compared with the central zones. This is obviously nutrition-related due to the better blood circulation in the peripheral areas. Furthermore, necrosis and cysts initially develop in the central zones of tumors, a phenomenon that is often observed macroscopically.

According to the in vivo PRs obtained in the present study, it can be concluded that in contrast to tumor stage (pT), tumor grade (G) exhibits a strong correlation with the individual PR. The individual tumor stage cannot be concluded from the PR, as tumor stage is a function of cell proliferation as well as of the time of tumor growth, with the latter parameter not being measurable at the time of diagnosis. Some large RCCs exhibited a low PR. These data explain the clinical observation in cases of RCC in which, despite a non-surgical approach, for various reasons almost a proliferative arrest is noted at long-term follow-up [19]. These in vivo data correspond to the PRs previously found in breast cancer by Lelle et al. [14]: a positive correlation between histological grading, recurrence rate and PRs was observed as well.

In conclusion, immunohistochemical determination of the tumor-specific PR using the monoclonal antibody Ki-67 is a practicable, reliable and reproducible method that complements conventional tumor staging and grading. Measurement of the individual PRs appears to be an additional diagnostic parameter for the detection of RCC patients at high risk, especially at early stages showing identical histological features.

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