*Advances in Brief*

# Genetic Aberrations Detected by Comparative Genomic Hybridization Are Associated with Clinical Outcome in Renal Cell Carcinoma[1]

## Holger Moch, Joseph C. Presti, Jr., Guido Sauter, Niels Buchholz, Paul Jordan, Michael J. Mihatsch, and Frederic M. Waldman[2]

*Division of Molecular Cytometry, Department of Laboratory Medicine [H. M., F. M. W.] and Department of Urology [J. C. P.], University of California San Francisco, San Francisco, California 94143-0808, and Institute of Pathology [G. S., M. J. M.], Clinic for Urology [N. B.], and University Computer Center [P. J.], University of Basel, 4003 Basel, Switzerland*

## Abstract

The clinical behavior of renal cell carcinoma (RCC) cannot be predicted by histological and other markers. In this study, comparative genomic hybridization was used to evaluate whether the number of genomic aberrations has prognostic significance in 41 nonmetastatic clear cell RCC extending beyond the renal capsule. Losses were most prevalent at 3p (56%) and 9p and 13q (24% each). The number of DNA losses per tumor was associated with recurrence-free survival ($P = 0.03$). DNA gains most often involved chromosome 5q (17%) and chromosome 7 (15%). The number of DNA gains was not associated with clinical outcome. Loss of chromosome 9p was the only individual locus associated with recurrence ($P = 0.04$), suggesting that a tumor suppressor gene on chromosome 9p may play a role in RCC progression.

## Introduction

The clinical outcome of RCC[3] varies considerably, especially in tumors presenting without metastases but extending beyond the renal capsule. The early identification of patients at higher risk for metastasis may prove useful because these patients may benefit most from aggressive adjuvant treatments. Recent studies characterizing genetic aberrations in RCC have implicated a number of chromosomal loci as important in cancer development and progression (1–4). However, the details of genetic changes in renal tumorigenesis are not well understood. It has been suggested that a net accumulation of genetic events is responsible for tumor progression in RCC (3, 4). In this study, CGH was applied to a set of nonpapillary RCCs to evaluate the prognostic significance of quantitative DNA aberrations in stage $T_3$, nonmetastatic RCC. CGH is based on a competitive *in situ* hybridization of differentially labeled DNAs, one from the tumor and another from the normal reference to normal metaphase spreads, allowing a survey of all DNA copy number changes (5).

## Materials and Methods

**Tumor Samples.** The tumor specimens were obtained from radical nephrectomies performed at the University of Basel (Basel, Switzerland) and at the Memorial Sloan-Kettering Cancer Center (New York, NY) between 1986 and 1991. Forty-one patients presenting with RCC extending beyond the renal capsule, and without evidence of metastasis at the time of surgery (pT3N0M0), were selected on the basis of nonpapillary clear cell histopathol-

ogy. Tumor material consisted of 22 fresh frozen tumors and 19 formalin-fixed tumor blocks. Histological grading was done according to the Fuhrmann system (6). Clinical follow-up data were available from 37 patients. These patients were monitored continuously with clinical examination, pulmonary X-ray, and computerized tomography. Recurrence-free survival was defined as the time between primary treatment and clinically detectable recurrent tumor.

Sections (5 $\mu$m) were cut from tumor blocks and stained with hematoxylin and eosin to ensure the histological representation of the sample. Specimens were trimmed as necessary by cutting normal tissue away with a scalpel.

For frozen material, DNA was extracted from eight 50-$\mu$m sections by proteinase K digestion (0.1 mg/ml) and phenol-chloroform extraction according to standard protocols. For paraffin material, twenty-five 10-$\mu$m sections were deparaffinized and suspended in DNA extraction buffer containing 0.5 mg/ml proteinase K. Additional proteinase K was added at 24 and 48 h later, for a total incubation time of 72 h (7). One $\mu$g of tumor DNA was nick translated by using a commercially available kit (BioNick kit; Life Technologies) for direct labeling with fluorescein-12-labeled dUTP (DuPont, Boston, MA). Normal reference DNA was extracted from mononuclear cells obtained from peripheral blood of healthy male or female volunteers and labeled as described above with Texas Red-5-labeled dUTP (DuPont).

**CGH and Digital Image Analysis.** CGH, image acquisition, and image processing were done as described previously (7). Fluorescein-labeled tumor DNA (200 ng), 200 ng of Texas Red-labeled reference DNA, and 20 $\mu$g of unlabeled human Cot-1 DNA (GIBCO) were hybridized to normal metaphase spreads. Hybridization was allowed to proceed for 2–3 days at 37°C in a moist chamber. Posthybridization washes were carried out as described previously (8).

A second repeat hybridization was done for all tumors using Texas Red-labeled tumor and FITC-labeled normal DNA to confirm the aberrations detected after the initial hybridization using FITC-labeled tumor DNA ("inverse" labeling CGH). Each CGH experiment included a control hybridization of FITC- and Texas Red-labeled normal DNAs to each other.

The digital image analysis system developed specifically for CGH has been described previously (5). In brief, images (4′,6-diamidino-2-phenylindole, FITC, and Texas Red) were collected from six to seven metaphases using a Photometrics cooled CCD camera (Microimager 1400; Xillix Technologies, Vancouver, British Columbia, Canada) and a SUN workstation. Green and red fluorescence images were acquired and analyzed separately. Green:red fluorescence intensity ratio profiles were calculated for each chromosome. The final results were expressed as the mean green:red ratio profile $\pm$ 1 SD determined from two to three metaphases (four observations/autosome and two observations/sex chromosome). Increased DNA sequence copy number was defined if an average ratio minus one SD was >1.0 on both usual and "inverse" CGH. Decreased copy number was defined if an average ratio plus one SD was <1.0 in both CGH reactions. Fifteen RCC specimens have been analyzed by both CGH and RFLP analysis at 18 loci (3). CGH and RFLP data demonstrated a high degree of concordance ($P < 0.001$).[4]

**Statistics.** The associations among the total number of DNA sequence copy number aberrations and outcome in groups with and without recurrence were evaluated using the Mann-Whitney $U$ test. The proportional hazards model was used to test whether there is a linear risk of disease recurrence with the total number of chromosomal aberrations. Recurrence-free survival rates were plot-

ted using the Kaplan-Meier method using the median number of aberrations as a cut point for grouping between the low and high number of aberrations. Statistical differences between the groups were determined with the log-rank test. The most common individual aberrations were studied for their association with recurrence-free survival using the Kaplan-Meier method. Twenty-four patients were censored at the time of their last clinical follow-up.

## Results

**Overview of Genetic Changes in RCC.** Thirty-seven of the 41 nonpapillary, clear cell RCC examined showed either relative loss (34 of 41) or relative gains (18 of 41) by CGH. Copy number aberrations are shown for the entire genome in Fig. 1. There was an average of 4.6 total aberrations/tumor (range, 0–18), 3.6 losses (range, 0–16), and 1.0 gains (range, 0–5). Losses were most frequently observed on chromosome 3 (56%). Fig. 2 shows the areas of chromosome 3 involved in DNA sequence copy number aberrations. Loss of the entire chromosome 3 was found in five cases. The minimal overlapping region of loss on chromosome 3 was 3p21–pter. DNA sequence losses involving chromosome 9 were present in 12 of 41 tumors (29%), involving the entire chromosome 9 in five cases, 9p alone in five cases, and 9q alone in an additional two cases. Other common aberrations included losses of 13q (24%), 6q (22%), and 8p, 14q, and
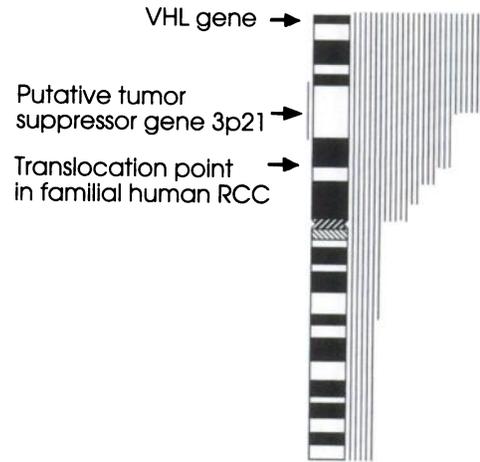


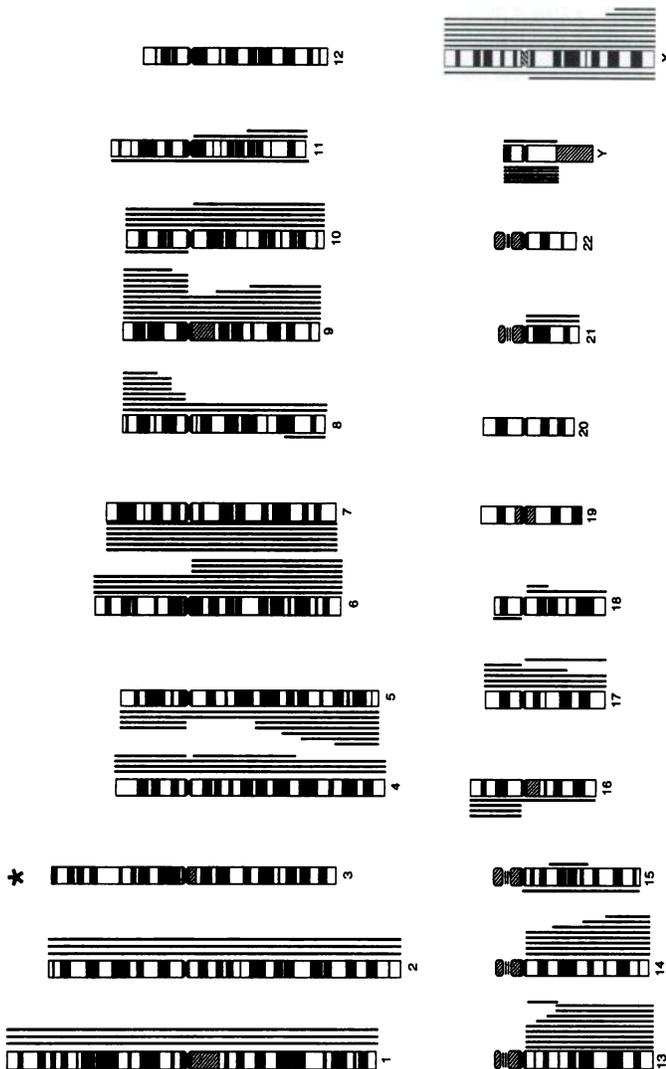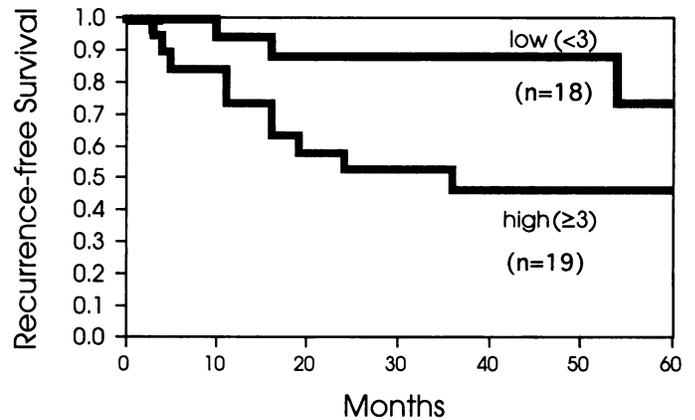Fig. 2. Losses of DNA sequences involving chromosome 3.



Fig. 3. Recurrence-free survival rates of patients with low (<3/tumor) and high (≥3/tumor) numbers of DNA sequence losses ($P = 0.03$, log-rank test).

Xq (all 20%) and gains at chromosome 5q (17%) and chromosome 7 (15%). No high-level DNA amplifications were seen.

**Clinical Outcome and CGH.** Clinical outcome was available for 37 patients, with a mean follow-up of 44 ± 31 months (median, 39 months). There were 3 grade I tumors, 12 grade II tumors, 19 grade III tumors, and 3 grade IV tumors. Recurrence was observed in 13 patients. Clinical outcome of patients with low-grade tumors (grades I and II) was not significantly different to patients with high-grade tumors (grades III and IV) in this tumor set ($P = 0.42$).

The mean number of losses per tumor was higher in tumors with subsequent recurrence (5.6 ± 4.4) than in tumors without recurrence (2.9 ± 3.2). However, this difference did not reach statistical significance ($P = 0.098$), possibly because of the small number of cases. Proportional hazards analysis indicates that the risk of tumor recurrence is related linearly to the number of DNA losses ($P = 0.05$), but it is not related to DNA gains or the total number of aberrations. Survival analysis was performed using the median number of losses as a cut point (three losses/tumor). Recurrence-free survival of patients with tumors having a high number of losses (more than or equal to three losses/tumor) were compared with tumors with low number of losses (less than three losses/tumor). Fifteen patients with low and nine patients with high numbers of DNA losses were free of disease at their last clinical follow-up. The analysis revealed a strong association between the high number of losses and the short time to recurrence ($P = 0.03$, log-rank test; Fig. 3). The total number of aberrations and the number of gains were not significantly associated with patient prognosis.
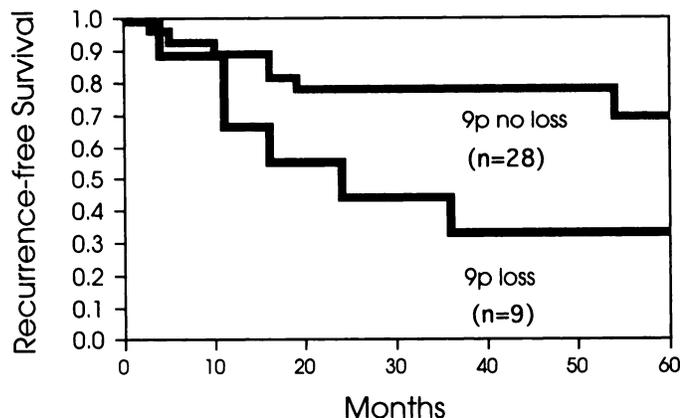


Fig. 1. Summary of all gains (*left*) and losses (*right*) of DNA sequences observed in 41 clear cell RCCs by CGH. Aberrations of chromosome 3 (*) are shown in Fig. 2.

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Fig. 4. Recurrence-free survival analysis for patients with and without loss of chromosome 9p ($P = 0.04$, log-rank test).

Once a significant association was seen between survival and CGH aberrations as a group, the four most common individual loci were tested to see which were most strongly linked. Chromosome 9p deletion was the only individual locus associated with clinical outcome. The recurrence-free survival of patients with 9p deletions was worse compared to patients having tumors without 9p deletions ($P = 0.04$, log-rank test; Fig. 4). Twenty-one patients without and three patients with 9p losses were free of disease at their last clinical follow-up. None of the other aberrations tested, including losses of 3p, was associated with prognosis.

## Discussion

It has been suggested that a net accumulation of genetic events is responsible for tumor progression and prognosis in RCC (3). The major aim of this study was to evaluate the prognostic significance of the number of genetic aberrations ("genetic grade") for patients with RCC. Our study showed an association between the total number of losses and overall survival, although there was no relationship with histological tumor grade or the number of gains. This observation is analogous to a result obtained by Isola et al. (8), who found a significantly worse prognosis in node-negative breast cancers with a high number of DNA losses compared to tumors with a low number of DNA losses. Because chromosomal deletions may inactivate tumor suppressor genes, our finding supports the hypothesis that inactivation of multiple suppressor genes underlies RCC progression .

The most frequent aberration found in this study was DNA sequence copy number loss involving chromosome 3p. This was expected because loss of the short arm of chromosome 3 represents the most common genetic aberration known in renal cancer (2–4, 9). However, 3p deletions were not predictive for clinical outcome. This is consistent with 3p deletion being an early event involved in tumor initiation rather than in tumor progression. At least three separate regions on chromosome 3 have been implicated by LOH studies in RCC: one coincident with the von Hippel-Lindau (VHL) disease gene locus at 3p25–26, one at 3p21–22, which may also be critical in small cell lung cancer, and one at 3p13–14, which includes the chromosomal translocation point in familial human RCC (10). The region of minimal deletion by CGH spans from 3p21 to 3pter (Fig. 2). Therefore, our data suggest that inactivation of both copies of either the VHL gene or the second putative tumor suppressor gene locus at 3p21–22 may be a critical event in tumorigenesis of RCC. Because VHL gene mutations have been found frequently in sporadic RCC, the VHL gene is the most likely target for a tumor suppressor gene in RCC.

There are few data available relating specific genetic events to malignant potential of renal cancer. The significant difference in recurrence-free survival between tumors with and without 9p deletion seen in this study suggests that a tumor suppressor gene on 9p is involved in RCC progression. Given the low number of examined cases and the number of variables tested, this result must be interpreted with caution. However, a role of a tumor suppressor gene on 9p for tumor progression would be consistent with recent findings in astrocytomas, implicating an association of 9p losses and tumor progression (11). Cairns et al. (12) have shown partial or complete deletion of chromosome 9 in 14 of 42 RCCs (33%). Recent evidence has implicated CDKN2 (MTS1 and p16) located at 9p21 as frequently aberrant in bladder and other solid tumors (13, 14). Because potential inactivation of CDKN2 by homozygous deletion, rearrangement, or point mutation was found to be rare in primary RCC (12), another gene at 9p21 may be the target of the observed deletions.

Other frequent sites of deletions included 6q, 8p, 13q, 14q, 17p, and Xq. The retinoblastoma (Rb) and p53 genes are located on chromosomes 13q and 17p, respectively. Both chromosomal loci are subject to allelic loss in RCC as determined by LOH studies (3, 4), although the rates of allelic loss reported vary considerably. Our data confirm a low frequency of 17p deletion in RCC. Losses on 13q were more common, but there was no association with prognosis in our tumor set. The 14q region has not been reported previously to be frequently deleted in renal cancer (4). However, our finding is consistent with cytogenetic reports showing frequent losses of the entire chromosome 14 (1). Monosomy 14 has been proposed as a marker for increased malignancy in RCC (15). In this patient set there was no association between 14q losses and tumor recurrence. It is interesting that chromosome 14 losses have been observed frequently by CGH in a small series of oncocytomas.[5]

LOH at 6q has been reported in various tumors, e.g., melanoma, ovarian, and breast cancer (16, 17). Because transfection of a normal human chromosome 6 suppresses the tumorigenicity of breast cancer cell lines, it was suggested that 6q might harbor a tumor suppressor gene. Our finding of frequent 6q losses by CGH is consistent with previous cytogenetic studies (15) and RFLP analysis (4), showing losses on 6q in 14 and 30%, respectively. These results suggest that a suppressor gene on 6q may also be involved in renal cancer.

The total number of regions with increased gene copy number was not associated with clinical outcome. This suggests that inactivation of tumor suppressor genes in several chromosomal sites, resulting in an aggressive tumor cell phenotype, seems to be more important in RCC development than oncogene amplification or occurrence of a few extra copies of DNA sequences at specific loci. This finding is consistent with a recent study by Isola et al. (8) who found no significant prognostic associations of increased DNA copy numbers in node-negative breast tumors. The overall absence of high-level amplifications and the low frequency of gains are in striking contrast to the extensive number of amplifications in breast or bladder cancer (18, 19) but comparable to prostate cancer (20). The absence of high-level amplifications is consistent with the absence of reports implicating oncogenes in kidney cancer and suggests that gene amplification is an uncommon mechanism for up-regulating expression of oncogenes in RCC. However, CGH is primarily a screening method that will not find very small (<100 kb) low-level amplifications and cannot detect activation of oncogenes due to other mechanisms than amplification (e.g., by a point mutation, transcriptional gene activation, or chromosome translocation).

Gains involving the long arm of chromosome 5q were observed in

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[5] Unpublished data.

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seven tumors (17%), whereas loss involving chromosome 5 was seen in only one tumor. In contrast, RFLP analysis has shown LOH on chromosome 5q in 30% of RCCs (4). In LOH studies using RFLP or microsatellite polymorphism, distinction between allelic gains and losses may be difficult. Our study suggests that the reported losses on 5q by RFLP may actually represent gains of the opposite allele. This finding is consistent with previous cytogenetic investigations (1, 2). It has been suggested that hematopoietic growth factor and receptor genes on the long arm of chromosome 5 (21) might be important in the development and progression of RCC (1).

Our data suggest that the total number of genomic losses (genetic grade) might have prognostic significance in patients with clear cell RCC. In addition, these results highlight several chromosomal regions that may harbor important genes for renal cancer. Frequent losses found by CGH in renal cancers included 9p and 13q, in addition to the locus reported previously on 3p. Losses of DNA sequences of 9p may be relevant for RCC progression.

## Acknowledgments

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