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Specific Loss of Chromosomes 1, 2, 6, 10, 13, 17, and 21 in Chromophobe Renal Cell Carcinomas Revealed by Comparative Genomic Hybridization

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We analyzed 19 chromophobe renal cell carcinomas by means of comparative genomic hybridization. Two tumors revealed no numerical abnormalities. In the remaining 17 cases we found loss of entire chromosomes with underrepresentation of chromosome 1 occurring in all 17 cases; loss of chromosomes 2, 10, and 13 in 16 cases; loss of chromosomes 6 and 21 in 15 tumors; and loss of chromosome 17 in 13 cases. The loss of the Y chromosome was observed in 6 of 13 tumors from male patients, whereas 1 X chromosome was lost in 3 of 4 tumors obtained from females. Comparative genomic hybridization results were verified by interphase cytogenetics. We conclude that a specific combination of multiple chromosomal losses characterizes chromophobe renal cell carcinomas and may help to differentiate them unequivocally from other types of kidney cancer. (Am J Pathol 1994, 145:356–364)

Recently, a new cytological type of human renal cell carcinoma (RCC) referred to as chromophobe RCC, which comprises approximately 5% of renal cell tumors, was described.[1] The unique morphological pattern of chromophobe cells was first recognized among experimentally induced renal tumors of the rat.[2] Chromophobe RCC is characterized by compact growth of tumor cells having a pale, fine reticular cytoplasm. The cytoplasm of chromophobe cells shows a diffuse reaction with Hale's acid iron colloid stain. Ultrastructurally, chromophobe cells display pathognomic cytoplasmic vesicles and a variable number of normal and morphologically altered mitochondria.[3,4] Only few reports describing the genetic changes of chromophobe RCCs have been published.[5–9] A recent restriction fragment length polymorphism (RFLP) analysis of chromophobe RCCs revealed a combination of allelic losses at 3p, 5q, and 17, which have not been seen in other genetic subtypes of kidney cancer and showed a gross rearrangement of the mitochondrial DNA.[7] A cytogenetic analysis of two chromophobe RCCs disclosed the common loss of chromosomes 1, 2, 6, 10, 13, 17, and 21 as well as loss of other chromosomes, yielding a low chromosome number of 34 and 38, respectively.[5,6] Two recent reports described an interstitial deletion at the short arm of chromosome 11 as the only karyotype change in a chromophobe RCC[8] and gains of chromosomes 7, 12, 16, 18, 19, and 20.[9]

In this study we used the new technique of comparative genomic hybridization (CGH).[10] Briefly, differently labeled tumor and normal genomic reference DNA are hybridized together to normal metaphase spreads under suppression conditions.[10–12] The ratio between the fluorescence intensities measured along each normal reference chromosome reflects the relative copy number of chromosomal segments in tumor cells, thus displaying relative overrepresentation and underrepresentation of chromosomal sequences in

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the tumor genome. The usefulness of the CGH technique to detect gross karyotype changes has been shown in several studies.[13–17] The sensitivity of CGH allows at present the detection of DNA gains and losses spanning at least 10 Mbp. In this study we have produced ratio fluorescence profile karyotypes from 19 chromophobe RCCs. Our report confirms highly specific combination of chromosomal losses that are associated with the development of this unique type of renal cancer.

Materials and Methods

Clinical and Pathological Data

Tumor tissue of 19 chromophobe RCCs was available for this study. Tumors were diagnosed according to the histological and cytological criteria described by Thoenes et al.[1,3] This series is comprised of tumors of 15 men and 4 women aged between 24 and 81 years (Table 1). The maximum diameter of tumors varied between 3.5 and 10 cm. All tumors were removed surgically, snap-frozen immediately in liquid nitrogen, and stored at −80 C until DNA was extracted.

Chromosome Preparations

Metaphase spreads for CGH experiments were prepared from phytohemagglutinin-stimulated lymphocytes of healthy individuals (46,XX or 46,XY) using standard procedures of hypotonic treatment and methanol/acetic acid fixation.

Labeling of DNA Probes

Total genomic DNA from tumor samples and regional DNA probes, recognizing tandem repetitive sequences in the paracentromeric regions of chromosomes 1 (pUC1.77)[18] and 3 (pa3.5),[19] were labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) or biotin-11-dUTP (Sigma, St. Louis, MO) using standard nick translation procedures.[20]

CGH

CGH experiments were performed as described previously with small modifications.[11,14] Briefly, approximately 100 to 200 ng of tumor DNA was hybridized together with the same amount of normal reference DNA in the presence of 50 µg Cot1-DNA and 50 µg sonicated salmon sperm DNA. *In situ* hybridization was allowed for 4 to 5 days. After posthybridization washes, biotin-labeled sequences were visualized using avidin conjugated to fluorescein isothiocyanate (FITC) and tetraethylrhodamine isothiocyanatae (TRITC)-conjugated sheep anti-mouse antibodies for the detection of digoxigenin-labeled sequences. DAPI banding was applied to identify individual chromosomes.

Fluorescence Microscopy and Digital Image Acquisition and Processing

A Zeiss Axiophot microscope with an aligned filter set for DAPI (LP 450–490, BP 365, FT 395), FITC (LP 515–565, BP 450–490, FT 510), and TRITC (LP 590, BP 546, FT 580) was used. For image acquisition the microscope was equipped with a cooled CCD camera (Photometrics; Kodak KAF, Tucson, AZ). The image acquisition procedure and image processing were performed as described.[11,14] Briefly, gray level images were taken separately for each fluorochrome and the DAPI image. Optimal exposure times and optical settings were chosen to avoid saturation values and to cover at least half of the total dynamic range of the camera.

The digital images were processed with programs developed in our laboratory (S du Manoir et al, manuscript submitted). Briefly, after subtraction of background fluorescence intensities and image shift correction an FITC to TRITC pixel-by-pixel ratio image was obtained by dividing an FITC image pixel by pixel by the TRITC image. A lookup table was used for the visualization of three different gray levels indicating the range below, within, or above the threshold of the normal range. Thus, DNA segments both within and outside the normal range within a single metaphase spread could be visualized.

To test for the consistency of values outside the normal range in several metaphase spreads, FITC to TRITC average ratio profiles were calculated. This was done by calculation of the medial axis of each chromosome within the DAPI image, calculation of FITC and TRITC profiles along individual chromosomes, and as a last step an averaging of individual chromosome ratio profiles from different metaphases. The central line in the profiles represents the most frequently measured fluorescence ratio for each reference metaphase spread, whereas the left and right vertical lines define emperically determined threshold values for underrepresentations and overrepresentations, respectively. The entire procedure will be described in detail elsewhere (S du Manoir et al, manuscript submitted).

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Interphase Cytogenetics

Interphase cytogenetics was performed on single cell suspensions from five tumors. Cells were incubated in 1X phosphate-buffered saline with 1% Tween 20 for 30 minutes at room temperature followed by an incubation in 1 M sodium thiocyanate at 70 C for 30 minutes.[21] After washing with distilled H_2O, digestion with pepsin (250 µg/ml in 0.2 N HCl) was conducted at 37 C for approximately 5 to 15 minutes. Postfixation was performed with 1% paraformaldehyde.

Criteria for the evaluation of FISH signals were as detailed in.[21] Evaluation was performed by counting at least 100 nuclei/slide. To minimize misinterpretations and to establish the accurate degree of ploidy, two-color hybridization experiments were performed with one probe testing for a chromosome present in apparently balanced copy number, and a second probe testing for the chromosome present in unbalanced numbers as revealed by cytogenetic analyses or CGH.

Results

CGH experiments were started with tumors 315, 277, and 229 because cytogenetic and/or RFLP analysis data were available for comparisons.[5,7] Cytogenetic analysis for tumor 315 had revealed the karyotype: 34, XY, −1, −2, −3, −6, −7, −9, −10, −12, −13, −17, −18, and −21.[5] Southern analysis using DNA probes from chromosomes 3, 5, and 17 revealed loss of constitutional heterozygosity (LOH) at the short arms of chromosomes 3 and 17.[7]

CGH with DNA from this tumor yielded fluorescence intensities over the autosomes at two different levels: the autosomes 1, 2, 3, 6, 7, 9, 10, 12, 13, 17, 18, and 21 were consistently less intensively painted than the remaining autosomes (Figure 1,A). In contrast, normal reference DNA painted all autosomes equally except for the constitutive heterochromatin, which showed very weak if any labeling with both tumor and reference DNA sequences. This effect was due to Cot1-DNA suppression of the hybridization of labeled sequences to highly repetitive target DNAs. The X chromosome yielded lesser fluorescence intensity values than autosomes since the reference DNA was male (Figure 1,B). A lookup table visualized the different FITC to TRITC fluorescence ratios (Figure 1,C): yellow represents a balanced FITC/TRITC ratio, whereas red represents a decreased FITC/TRITC ratio.

For tumor 277 banding analysis had revealed the karyotype: 37, X, −Y, −1, −2, −6, −10, −13, −15, −17, and −21.[6] As in tumor 315 two ranges of fluorescence intensity ratios could be distinguished over the reference chromosomes. The less intensively painted chromosomes again corresponded to the monosomies of the banding analysis, except for the monosomy 15 which could not be verified by CGH (Fig. 2,B). An average ratio profile from 10 metaphase spreads confirmed unequivocally the occurrence of only two different fluorescence ratio levels (Figure 3).

RFLP analysis of tumor 229 revealed LOH for DNA markers on the long arm of chromosome 17 but not on chromosomes 3p and 5q.[7] CGH revealed that chromosome 17 was one of the less intensively painted chromosomes, whereas chromosomes 3 and 5 were more intensively painted (Table 1).

In summary, CGH findings for these three tumors, with the exception of a single chromosome in one case, were evaluated in complete accordance with previously established cytogenetic and molecular genetic data. To test for the consistency of chromosome losses, we evaluated a series of other chromophobe RCCs that had not been studied before. Figure 2,D shows a representative example of CGH with DNA from tumor 691. Again the two different ranges of fluorescence values with chromosomes 1, 2, 6, 10, 13, and 21 painted less intensively were observed.

Underrepresentations of multiple chromosomes were found in 17 tumor DNA samples (Table 1, Figure 4). Only in DNA samples from two tumors revealed CGH a uniform staining over all autosomes without any consistent overrepresentation or underrepresentation of chromosomal regions. Entire chromosomes were lost in all but one case; in tumor 680 a loss was indicated only for the short arm of chromosome 1. Underrepresentation of chromosome 1 was found in each other tumor; underrepresentation of chromosomes 2, 10, and 13 occurred in 16 cases each; loss of chromosomes 6 and 21 in 15 tumors; and loss of chromosome 17 in 13 tumors. The losses of chromosomes 3 and 8, chromosomes 9 and 18, and chromosomes 4, 5, 12, 14, 15, and 20 occurred in 4, 3, and 1 cases, respectively. The loss of the Y chromosome was observed in 6 of 13 tumors from males, whereas one of the X chromosomes was lost in 3 of 4 tumors from females. Thus, seven chromosomes, ie, chromosomes 1, 2, 6, 10, 13, 17, and 21, were specifically involved in copy number changes. The loss of all the seven chromosomes occurred in 10 of 17 cases, monosomy of six specific chromosomes in 4 cases, monosomy of five chromosomes in 2 cases, and four chromosomes in 1 case.

Because CGH provides only data for relative gains and losses of chromosomes but not for the actual ploidy, interphase cytogenetics was performed on single cell suspensions from several tumors (Table 2).

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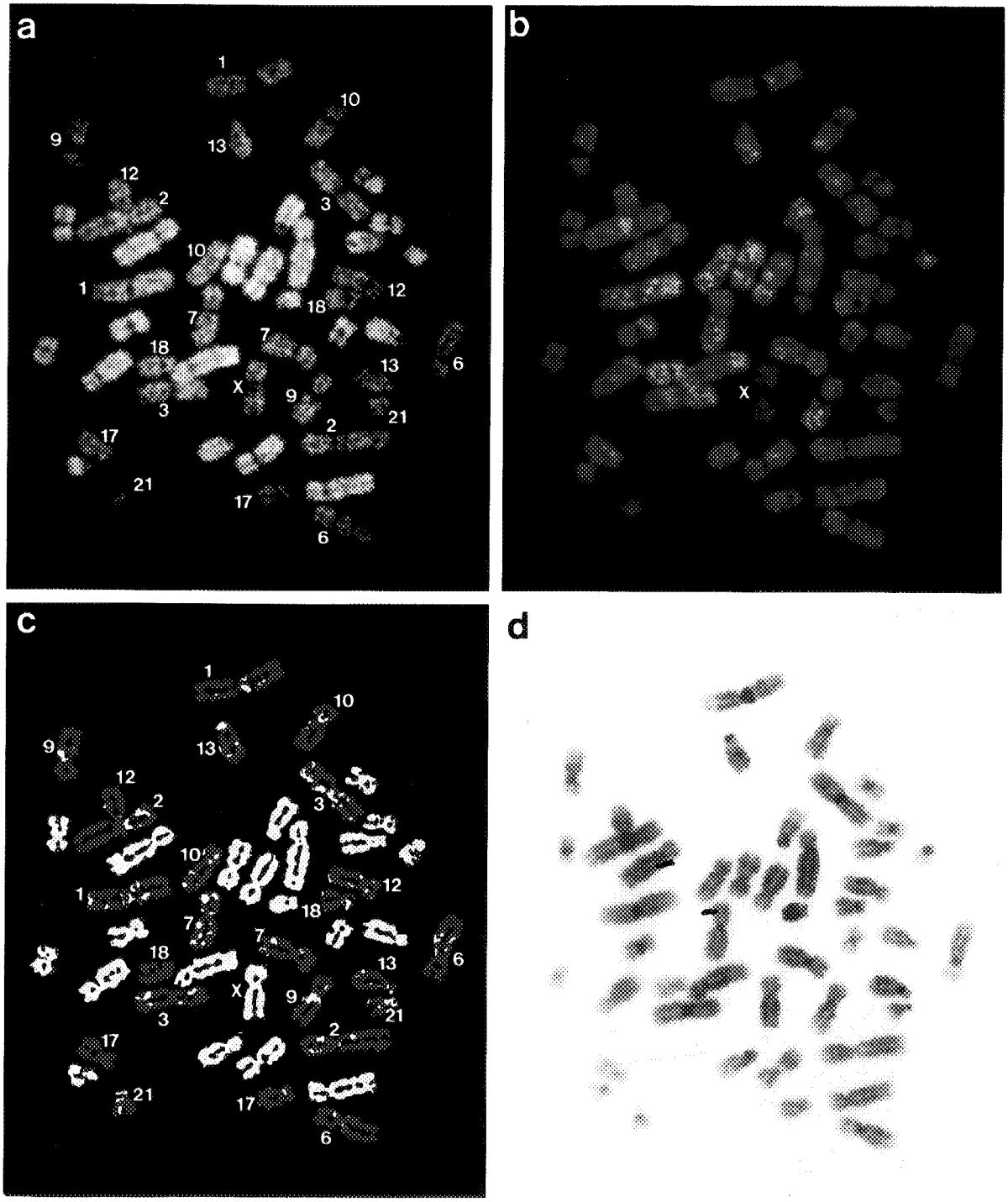

Figure 1. a-d: *CCD images of a male metaphase spread hybridized with tumor DNA 315 detected with FITC* (a) *and (46,XY) control DNA detected with TRITC* (b). *The autosomes in* a *reveal two different fluorescence intensity levels: chromosomes 1, 2, 3, 6, 7, 9, 10, 12, 13, 17, 18, and 21 yield lower intensity values that are equal to the fluorescence intensity values of the X chromosome (male patient). A lookup table visualizes the pixel-by-pixel FITC/TRITC ratios in* c. *Yellow suggests a balanced state between tumor DNA and the male control DNA, whereas red indicates an underrepresentation of tumor DNA. For chromosome identification DAPI banding was applied* d. *The karyotype of this tumor was published previously in (5).*

pUC1.77 and pa3.5, which recognize the highly repetitive sequences in the centromeric regions of chromosomes 1 and 3, respectively, were chosen because chromosome 1 yielded a decreased FITC/TRITC ratio, indicating an underrepresentation of chromosome 1, whereas chromosome 3 showed a balanced FITC/TRITC ratio. The hybridization efficiency of the probes was tested on kidney cells from

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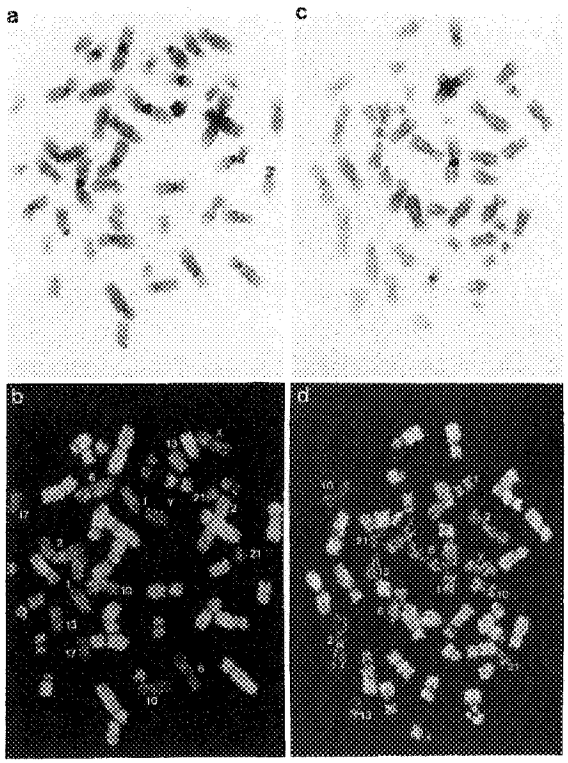

Figure 2. a-b: *CCD images of a male metaphase spread hybridized with tumor DNA 277 detected with FITC* b. *The hybridization pattern of the (46,XY) control DNA is not shown.* b *demonstrates again the two different fluorescence intensity levels observed in chromophobe RCC: autosomes 1, 2, 6, 10, 13, 17, and 21 are less intensively painted and display the same fluorescence intensities as the X chromosome (male patient). Note that the Y chromosome is not painted at all, indicating a loss of this chromosome in the tumor.* a *DAPI image for identification of the chromosomes. The karyotype of this tumor was published in (6).* c-d: *CCD images of a female metaphase spread.* d *displays the FITC image of the hybridization pattern with the tumor DNA 691; the TRITC image of the control DNA is not shown. Again the typical two level fluorescence intensity pattern for chromophobe RCC is visible, indicating loss of autosomes 1, 2, 6, 10, 13, and 21 with fluorescence intensities equal to the X chromosomes (male patient).* c *DAPI image.*

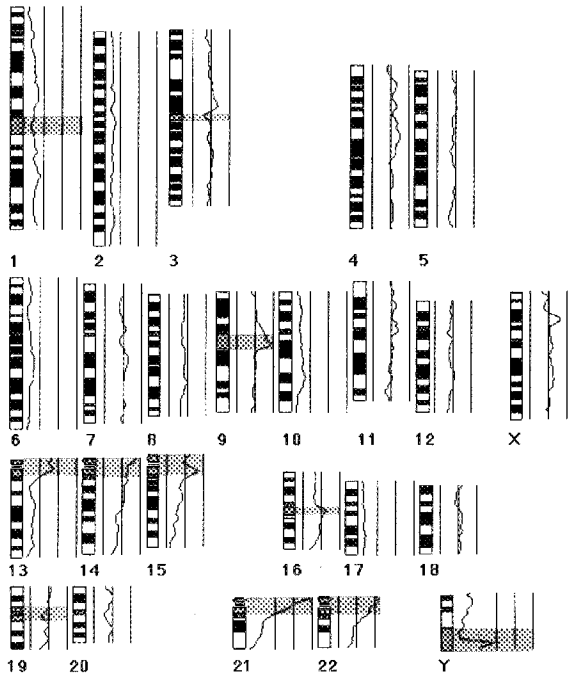

Figure 3. *Average fluorescence ratio profile for tumor 277. The central vertical line represents the ratio value typical for a balanced state of chromosome material, whereas the right and left lines represent thresholds for chromosomal gains (right line) and losses (left line). Gray-shaded regions indicate heterochromatic pericentromeric and paracentromeric chromosome regions and the short arms of the acrocentric chromosomes that yield due to the suppression with Cot-1 DNA very low FITC and TRITC fluorescence intensities and are therefore excluded from evaluation.*

normal kidney tissue (Table 2, 9759/93). Two signals were counted for both probes in more than 90% of the nuclei. In contrast, in all tumor samples the chromosome 1-specific probe pUC1.77 revealed only one signal in the majority of cells (78 to 89%), whereas the chromosome 3-specific probe pa3.5 yielded two signals in 89% to 91% of cells. Taking into account the average ratio profiles that showed unequivocally that only two different FITC/TRITC ratio levels existed (Figure 3), we concluded that chromosomes with a decreased FITC/TRITC ratio corresponded to monosomies and the other chromosomes to disomies.

Discussion

In this study we used the recently introduced CGH technique[10,11] to analyze 19 chromophobe RCCs. Our data confirm previous cytogenetic data that these tumors are characterized by a combination of multiple losses of the entire chromosomes 1, 2, 6, 10, 13, 17, and 21.[5,6]

The recurrent loss of a set of 6 or 7 entire chromosomes in the vast majority of chromophobe RCCs is a unique genetic pattern. In contrast, the vast majority of solid tumors are characterized by complex karyotype changes such as monosomies, trisomies, interstitial deletions, and translocations leading to loss, gains, and rearrangements of different chromosomal segments. For example, nonpapillary RCC, the most common type of kidney cancer, has either one, two, three, or more specific karyotype changes including deletions, monosomies, trisomies, and balanced or unbalanced translocations.[22]

It is likely that multiple hits represent sequential mutations of growth regulatory genes in a single clone. The loss of chromosome 1 occurred in all chromophobe RCCs where numerical chromosomal changes were detected, whereas other chromosomes were lost only in a fraction of the tumors studied so far. This finding suggests that chromosome 1 harbors one or several genes whose loss is a necessary condition for the development of chromo-

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Table 1. *Whole Chromosome Losses Revealed with Comparative Genomic Hybridization*

File No.	Sex	Age	Size of Tumor (*cm*)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
224	F	NK	1.0	*	*				*				*			*				*			*	*			
229†‡	F	24	3.5	*	*				*				*			*				*	*			*		*	
277†	M	58	4.5	*	*				*				*			*				*				*			*
315†§	M	63	6.0	*	*	*			*	*		*	*		*	*				*	*			*			
417	M	NK	1.0	*	*	*		*	*		*	*	*			*				*				*			*
680	M	61	5.5	*		*	*		*		*		*			*	*	*			*			*			*
685	M	51	7.5	*	*				*				*			*				*				*			
686	M	69	10.0	*	*				*				*			*				*				*			*
687	M	60	8.0	*	*				*				*			*				*				*			
689	M	43	8.0	*	*				*				*			*				*				*			
690	F	81	8.5	*	*				*	*	*		*			*				*				*		*	
691	M	42	5.0	*	*				*				*			*								*			
692	M	63	4.7	*	*				*				*			*								*			
693†	M	49	8.6	*	*	*			*							*				*				*			
694	F	57	4.3	*	*							*	*							*				*		*	
695	M	69	7.0	*	*								*			*											*
696	M	60	4.3	*	*				*		*		*			*				*							*

F, female; M, male; NK, not known.
* Loss of one of the homologous autosomes or one of the sex chromosomes.
Previously published in: †6; ‡7; §5.

phobe RCCs. However, loss of tumor-relevant genes on chromosome 1 does not seem to be a sufficient condition because in all tumors studied thus far a series of specific chromosomes was lost. We expect that chromosomes 2, 6, 10, 13, 17, and 21 should harbor additional tumor-relevant genes that also contribute to the formation of this tumor entity when present in diminished copy number. It seems possible that mutations of these genes occurred in the cases in which we observed a balanced state of the respective chromosomes by CGH. Alternatively, this finding could suggest that the loss of the entire set of tumor-relevant genes located on other chromosomes than chromosome 1 is not absolutely essential for chromophobe RCCs. Whether a specific order of chromosome losses is required or whether the order that leads to the loss of a specific set of chromosomes may be random cannot be deduced from these data.

A strong increase of chromosome segregation errors may be the primary event in the development of chromophobe RCCs.[23] Gains or losses may affect all chromosomes similarly. Selection due to growth advantages of progenitor cells with specific chromosome losses could then explain why a specific pattern of chromosome losses is found in fully developed chromophobe RCCs. Currently, we can only speculate about the possible genetic events that lead to the unique pattern of chromosomal losses in this tumor type.

Errors in the interaction between mitochondrial and nuclear genes could also be instrumental in the development of chromophobe RCCs because gross rearrangement of the mitochondrial DNA in chromophobe RCCs was previously described.[7]

Chromophobe RCC may pose a differential diagnostic problem by routine histological examination. Thoenes et al[3] have recognized tumors with a high number of vesicles (typical variant) and those with a high number of mitochondria (eosinophilic variant). A transition between the two types is a common finding within chromophobe RCCs. The transparent cytoplasm of normal variant may resemble "clear" cells and may be misdiagnosed as a clear cell RCC. The mitochondria-rich variant of chromophobe RCC, which is characterized by dense eosinophilic granular cytoplasm, may resemble a benign oncocytoma or malignant granular cell RCC. Recent working classifications are based on cytological and histological pattern of renal cell tumors. In regard to certain cytoplasmic components, the World Health Organization describes clear, granular, oncocytic, and spindel-shaped cell tumors.[24,25] Thoenes et al[26] distinguishes clear (typical and eosinophilic variant), chromophilic (eosinophilic and basophilic variant), chromophobe (typical and eosinophilic variant), pleomorphic, and oncocytic cells. Such stratifications based solely on variable cellular phenotypes may not be sufficient to discriminate between genetically distinct entities.

Recent molecular cytogenetic evaluation of renal cell tumors has recognized genetically and biologically well-defined entities referred to as papillary renal cell adenoma and carcinoma, nonpapillary RCC, and renal oncocytoma.[22,27] We have now established the genetic hallmarks for chromophobe RCCs as well. Each type of kidney cancer is characterized by specific combinations of genetic changes and some of them occur exclusively in one of the tumor types.[22]

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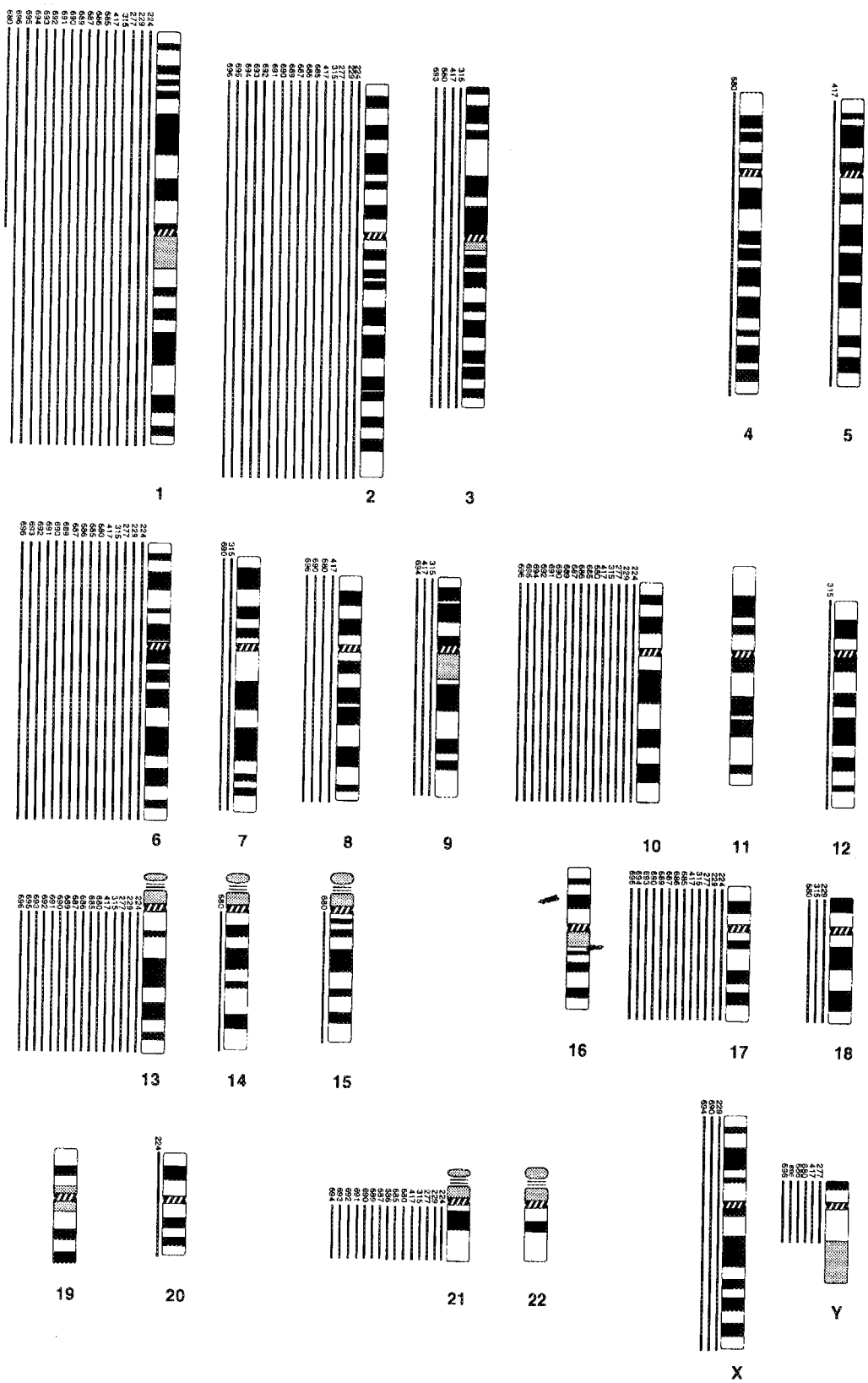

Figure 4. *Summary of all gains and losses found with CGH. Vertical lines on the right side of a chromosome represent gain of genetic material, whereas vertical lines on the left side correspond to losses. Case numbers are provided on the top of each line. Gains were not detected in any tumor.*

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Table 2. *Results of Interphase Cytogenetics*

File No.	DNA Probe	No. of Signals/Cell					No. Nuclei
		0	1	2	3	4	
9759/93	pUC1.77		5	92	3		100
	pa3.5		5	93	2		100
686	pUC1.77	1	89	12			102
	pa3.5	1	2	89	7	3	102
687	pUC1.77	1	87	12			100
	pa3.5		2	91	2	5	100
689	pUC1.77	3	78	19			100
	pa3.5		2	89	9		100
691	pUC1.77	3	87	13			103
	pa3.5		1	91	2	9	103

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