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Molecular Cytogenetic Analysis of 17 Renal Cancer Cell Lines: Increased Copy Number at 5q31-33 in Cell Lines from Nonpapillary Carcinomas

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Comparative genomic hybridization (CGH) was used to screen for genomic imbalances in cell lines derived from 13 nonpapillary renal-cell carcinomas (RCCs), two papillary RCCs, one renal squamous-cell carcinoma, and one transitional-cell carcinoma of the renal pelvis. Aberrations were found in all 17 lines. The most frequent changes in nonpapillary RCC cell lines were gains of 5q (85%), 7q (69%), 8q (69%) and 1q (54%) and losses of 3p (92%), 8p (77%), 4q (62%) and 14q (54%). High-level gains (HLGs) were detected at 4q12, 5p, 5q23-33, 7q22-qter, 8q23-24, 10q21-qter, 12p and 12q13-22. By means of fluorescence *in situ* hybridization (FISH) we narrowed the smallest common region involving 5q gains to the genomic segment between D5S642 and D5S673, and found that the HLG at 4q12 possibly involved amplifications of *c-kit* and *PDGFRA*. Two papillary RCC cell lines showed gains of entire chromosomes 7, 12 and 17. The CGH data reported here should help to facilitate the choice of individual renal-tumor cell lines for exploring target genes in regions of interest.

Key words: Renal cell carcinoma (RCC) — CGH — FISH — Gene amplification

Renal carcinomas comprise a heterogeneous group of tumors that together account for 2–3% of all cancers in adults.[1)] Renal-cell carcinoma (RCC), which originates in the renal cortex, accounts for 80–85% of primary malignancies of the kidney.[2)] Cytogenetic and molecular genetic studies separate RCC into two types: nonpapillary tumors characterized by abnormalities of chromosome 3p and papillary tumors, which do not show 3p abnormalities.[3)] Other histologic types, including transitional cell carcinoma (TCC) of the renal pelvis, make up the remaining 15–20% of renal cancers in adults.[4)]

Cytogenetic studies have implicated a number of chromosomal loci as important in development and progression of RCC.[3)] As with other solid tumors, an accumulation of genetic events may be responsible for tumor progression in the kidney.[5, 6)] However, the genetic changes that occur in renal tumorigenesis are not well understood, because the karyotypes of RCCs and their cell lines are extremely complex and involve several poorly characterized marker chromosomes.

Comparative genomic hybridization (CGH) permits analysis of DNA copy-number gains and losses across the entire genome in a single hybridization experiment.[7)] Theoretically, all DNA copy-number aberrations (CNAs) spanning two megabases (Mb) or more can be detected by CGH[8)]; moreover, chromosomal regions showing high-level gains (HLG) indicative of gene amplification can provide useful targets for positional cloning of proto-oncogenes.[9–12)]

Here we report patterns of gains, losses, and amplifications of chromosomal material among 17 renal cancer-derived cell lines consisting of 15 RCCs, one renal squamous-cell carcinoma (SCC), and one renal TCC, achieved by CGH and fluorescence *in situ* hybridization (FISH) together with standard Q-banding. Immortalized RCC cell lines provide an inexhaustible source of DNA and RNA, as well as models for biological and functional studies designed to provide information essential for cloning genes involved in development and progression of this type of cancer.

MATERIALS AND METHODS

Cell lines A total of 17 cell lines used in the present study were histopathologically classified into four types: 13 nonpapillary RCC lines, two papillary lines, one SCC line and one TCC line. Among these, eight nonpapillary lines (RCC19, -23, -31, -149, -213, -217, -270, -826), one papillary line (RCC266), one SCC line (RSC261) and one TCC line (RTC274) were established in our laboratory from primary tumor tissues, which were kindly provided by Dr. H. Oshima (Tokyo Medical and Dental University), Dr. M. Wakisaka (Chiba University), Dr. I. Fukui (Cancer Institute). Two lines, HsBt and HOKN9 were provided by Dr. Y. Fujii (Hasumi Electro-Chemical and Cancer Institute), KU-Ep by Dr. M. Tachibana (Keio University), KC12 by Dr. M. Oshimura (Tottori University), and C6-3 by Dr. T.

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Shuin (Kochi Medical College). The remaining papillary ACHN line was purchased from Dainippon Pharmaceutical Co. (Osaka). The RCC19 line came from a tumor with sarcomatoid transformation. The histological classifications, stages and grades of tumors employed for establishment of the nonpapillary RCC cell lines are listed in Table I. Clinicopathological classifications were made according to the "General Rule for Clinical and Pathological Studies on Renal Cell Carcinoma" published jointly by the Japanese Urological Association, the Japanese Society of Pathology, and the Japan Radiological Society.[13] Cells were cultured in Dulbecco's minimal essential medium (DMEM) in Eagle's balanced salt solution supplemented with 10% fetal calf serum, at 37°C in a 5% CO_2 atmosphere. High-molecular-weight DNA was isolated from each cell line and, for reference, from peripheral lymphocytes of a normal male volunteer.[10, 12]

Chromosome preparations and Q-banding Chromosomes were prepared according to standard methods and analyzed by Q-banding as previously described.[14] Karyotypes were determined according to the International System for Cytogenetic Nomenclature.[15]

CGH and digital imaging CGH was performed with directly fluorochrome-conjugated DNA.[10] In brief, the DNA of each cell line was labeled with Spectrum Green-dUTP (Vysis, Chicago, IL), and normal (reference) DNA was labeled with Spectrum Red-dUTP (Vysis), by nick translation. Labeled tumor and normal DNAs (200 ng each), together with 10 μg Cot-1 DNA (Gibco BRL, Gaithersburg, MD) were denatured at 70°C for 5 min in a 10 μl hybridization solution (50% formamide, 10% dextran sulfate, and 2× SSC), then hybridized to normal male metaphase chromosomes at 37°C for 48–72 h. The slides were washed for 2 min in 0.4× SSC/0.3% NP-40 at 75°C, then for 2 min at room temperature in 2× SSC/0.1% NP-40. Air-dried slides were counterstained with 0.1 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI).

Metaphase chromosomes were captured using an epifluorescence microscope (Olympus, Tokyo) connected to a cooled charge-coupled device (CCD) camera (Photometrics, Tucson, AZ). Three-color digital images were collected from each hybridization, in 5–10 metaphase cells, and processed with Vysis/Quips CGH software (Ver. 2.1). Chromosomal regions were interpreted as over-represented (gain) if a ratio of green to red >1.2 was observed, whereas regions with a green-to-red ratio <0.8 were interpreted as under-represented (loss). Any region in which the ratio of green to red was >1.5 we interpreted as HLG indicative of gene amplification.[7, 8, 10] Heterochromatic regions near the centromeres and telomeres, and the entire Y chromosome, were excluded from analysis.

FISH and chromosome painting Because we detected

Table I. Clinical Characteristics and Karyotype Abnormalities in 13 Nonpapillary RCCs

No.	Sex	Age	Stage	Cell type	Histological pattern	Chromosomal no. mode (range)	Clonal chromosome abnormalities
19	M	45	IV	Spindle	Sarcomatoid	82 (79–161)	del(1p), del(1q), der(3)t(3p;5q), del(9p), der(9)t(9p;?)
23	F	56	III	Clear	Alveolar	46 (44–93)	+X, der(1)t(1p;8q), der(3)t(3p;8q), +5, +7, del(8q), −13, −14, −21
31	M	52	IV	Clear	Unknown	62 (56–66)	der(1)t(1p;?), der(3)t(3p;5q), del(6q), der(9)t(9p;?), der(14)t(14p;?), der(17)t(1q;17q)
149	F	72	IV	Clear	Alveolar	85 (45–88)	der(1)t(1p;?), der(3)t(3p;5q), −5, −8, −9, −19, der(19)t(19p;?)
213	M	80	III	Clear	Alveolar/tubular	45 (38–82)	der(3)t(1q;3p), −4, +7, −8, del(9p), −14, i(18q), −19
217	M	58	IV	Clear	Unknown	76 (42–77)	del(1p), der(1)t(1q;?), der(2)t(2q;?), der(3)t(3p;5q), −5, der(6)t(6q;?), i(8q), −9, der(12)t(12q;?), −13, −14, −15, −17, −18, −19, −20
270	F	58	—	Clear	Unknown	72 (70–72)	der(3)t(3p;5q)
826	M	62	IV	Clear	Unknown	38 (31–83)	del(1q), t(1p;6q), t(2p;7p), der(3)t(3p;8q), del(8p), der(8)t(3p;8q;?), del(12q), del(14q)
HsBt[a]	M	57	I	Clear	Alveolar/tubular		
HOKN9[a]	M	61	I	Clear/spindle	Alveolar		
KC12[b]	M	44	—	—	—	59 (54–63)	+X, −Y, +1, +2, der(3)t(3p;5q), +5, +8, +10, +11, +12, +16, +17, +20, +21
KU-Ep	M	65	IV	Clear/granular	Alveolar	117 (92–147)	−4, i(5p), +6, +7, i(8q), +9, +20
C6-3	—	—	—	—	—	—	Unknown

a) Karyotypes of HsBt and HOKN9 will be reported in detail elsewhere.
b) See Kohno *et al.*[34]

frequent copy-number-gains (CNGs) at 5q and a remarkable HLG at 4q12 by CGH, we carried out FISH with whole-chromosome painting (WCP) 5 probe (Vysis, Downers Grove, IL) and yeast artificial chromosomes (YACs) on 5q or 4q12 to ascertain the structural and numerical bases of these aberrations. YAC clones were isolated from the Centre d'Etude du Polymorphisme Humain (CEPH) YAC library and their locations on the genetic map were compiled from the YAC information archived by the Whitehead Institute/MIT Genome Center (http//www-genome.wi.mit.edu/) and by Resources for Human Molecular Cytogenetics (http://bioserver.uniba.it/fish/rocchi/welcome.html). To define the minimal common region of gains on 5q23-q33 among three of the nonpapillary cell lines (RCC149, HOKN9, and KU-Ep), we examined the number of FISH signals specific for each probe in 50 nuclei of each cell line. Chromosome-painting combined with DAPI banding was performed with the WCP5 probe (Vysis) according to the manufacturer's instructions. FISH probes for YACs were generated by Alu-polymerase chain reaction (PCR) as described elsewhere.[16, 17]

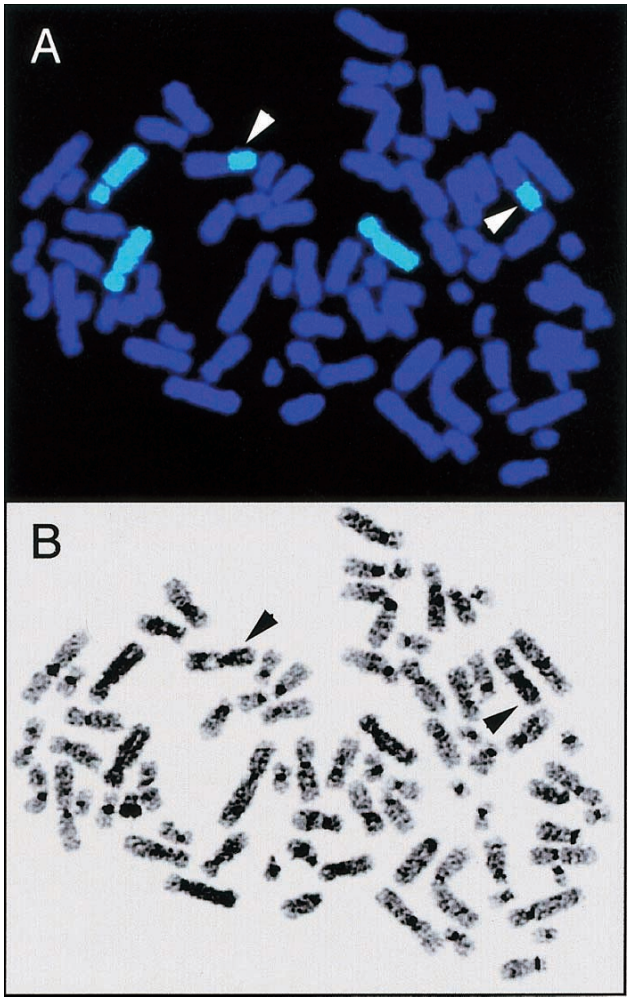

Fig. 1. (A) FISH of RCC19 with a whole-chromosome-painting 5 probe. (B) Banded chromosomes of the same metaphase spread shown in A. DAPI banding patterns were electronically visualized as black and white, for chromosome identification. Arrows indicate derivative chromosomes carrying unbalanced translocations between 3p and 5q.

RESULTS

Cytogenetic analysis Cytogenetic findings for the 12 nonpapillary RCC cell lines that were analyzed successfully in this manner are summarized in Table I. The modal chromosome numbers ranged from near-diploid to near-pentaploid. The most consistent and recurrent abnormalities were deletions or rearrangements involving chromosome 3p, seen in ten of the cell lines (83%). Karyotyping together with painting of chromosome 5 (Fig. 1) showed that six of the 3p deletions (in RCC19, -31, -149, -217, -270 and -KC12) had resulted from unbalanced translocations between 3p and 5q. Cell lines RCC23 and 826 each carried t(3p;8q).

Overview of DNA CNAs CNAs were found in all the cell lines examined. The average number of CNAs in nonpapillary RCCs was 14.4 per cell line (range, 7–23); the average number of gains was 8 (range, 4–11) and of losses, 6.4 (range, 2–13). CNAs in all 15 of the papillary and nonpapillary RCC cell lines analyzed are summarized in Fig. 2. The minimal common regions of gains seen most frequently in the nonpapillary RCC cell lines were at 1q23-32 (7/13; 54%), 2q22-24 (6/13; 46%), 3q22-25 (6/13; 46%), 5q31-33 (11/13; 85%), 7p11-15 (7/13; 54%), 7q31-35 (9/13; 69%), 8q24 (8/13; 62%), 12q15-22 (6/13; 46%), and Xq21-22 (6/13; 46%). The minimal common regions involved in losses were at 3p24-25 (11/13; 85%), 4q11-31 (8/13; 62%), 8p11-12 and 8p21-22 (9/13 each; 69%), 14q23-32 (7/13; 54%), and 18p and q (6/13 each; 46%). The smallest regions of HLG overlap were seen at 4q12 (one case, Fig. 3A), 5p (three cases), 5q23-33 (two cases, Fig. 3C), 7q22-qter (one case), 8q23-24 (four cases), 10q21-qter (one case), 12p (one case) and 12q13-22 (two cases). Gains involving whole chromosomes 7, 12 and 17 were identified in two papillary RCC cell lines. No losses of DNA or HLGs were seen in the papillary RCC lines. On the other hand, the SCC line (RSC261) exhibited gains of chromosomes 5p (HLG), 7, 11q13-14, 12, 14q, 15q, 19q, 20p12-13, 20q13, 21q22, 22q and Xq, and losses of chromosomes 3p, 4, 5q11-23, 11p, 17p and Xp. In the TCC line (RTC274), we documented gains of chromosomes 2p, 8q (HLG), 9q, 11p13-15, 16q, 17q, 20p and 21q, and losses of material on chromosomes 6, 8p, 9p and 16p13.

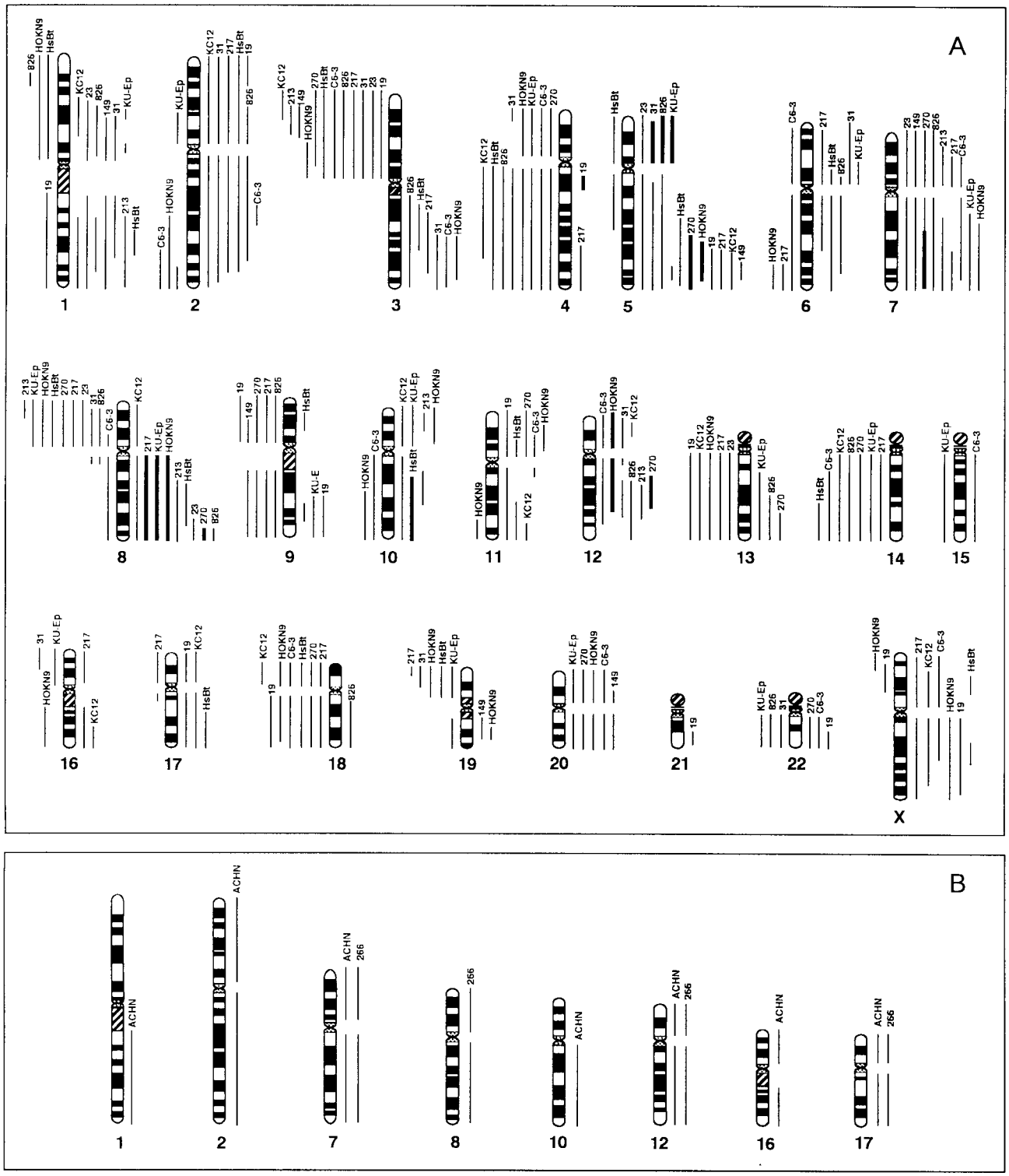

Fig. 2. Summary of all chromosomal gains and losses detected among 13 nonpapillary RCC cell lines (A) and 2 papillary RCC cell lines (B). Vertical lines on the right side of a chromosome indicate gains of genetic material, while those on the left side indicate losses. Thick lines represent high-level amplification.

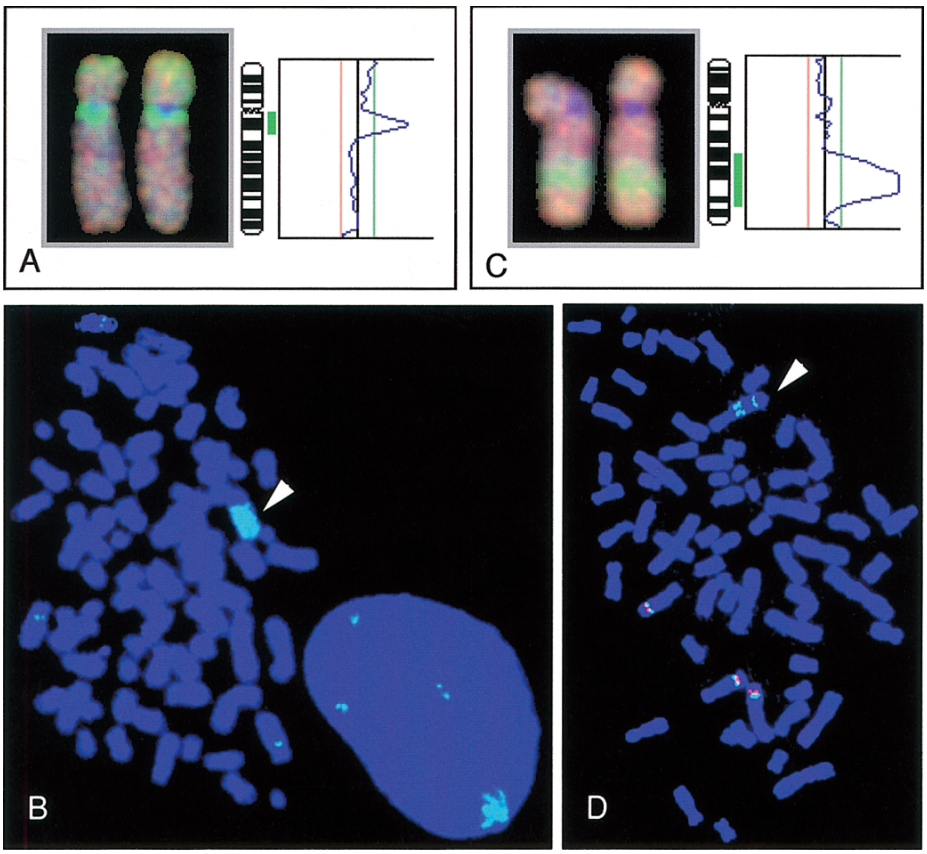

Fig. 3. (A) CGH images of chromosome 4 and the corresponding green-to-red profiles of RCC19, illustrating high-level amplification at 4q12. (B) FISH with YAC 944C7 (containing *c-kit*) showed strong signals indicative of gene amplification on both metaphase and interphase cells of RCC19. *c-kit* was amplified on the HSR of a marker chromosome (arrow) in this cell line. (C) CGH images of chromosome 5 and an average green-to-red profile in cell line HOKN9. (D) Representative two-color FISH of an HOKN9 cell hybridized to YACs 917H2 (D5S638; green) and 782B10 (D5S673; red). Six twin-spot green signals and three twin-spot red signals were detected in this metaphase spread. An arrow indicates one marker chromosome that harbors triplicate twin-spot signals specific for YAC 917H2, without any red signal. In the CGH profiles of control cells, the mean green:red ratio and the corresponding SD for all autosomes remained between 0.8 (red) and 1.2 (green).

Definition of the smallest common region of 5q gains by FISH FISH was performed with 20 YACs mapped within 5q13-34, to determine the smallest common region of overlapping 5q gains among RCC149, HOKN9, and KU-Ep. In the CGH experiments these three lines had exhibited much smaller regions of 5q gain than the other cell lines (Fig. 2A). As shown in Fig. 3D, in HOKN9 one marker chromosome exhibited triplicated twin-spot signals for YACs on 5q23-q32, from D5S494 (YAC 818C10) to D5S638 (YAC917H2), but no YAC proximal or distal to this region was amplified on the marker chromosome. Moreover, in both RCC149 and KU-Ep, the average number of signals per nucleus for YACs distal to D5S658 (mean±SD; RCC149=6.4±1.2, KU-Ep=4.9±1.6) was higher than that found for YACs proximal to D5S642 (RCC149=4.1±1.4, KU-Ep=3.7±1.7). These results indicated that the minimal region covered by overlapping 5q gains among these three cell lines lay between D5S642 and D5S673 (Fig. 4).

Validation of increased copy number at 4q12 in RCC19 FISH on metaphase cells of RCC19 with YACs 944C7 and 972B3, which contain *c-kit* and *PDGFRA* genes respectively, showed strong signals as a homogeneously staining region (HSR) on the long arm of a marker chromosome (Fig. 3B).

DISCUSSION

By CGH analysis we determined that loss of chromosome 3p was the most frequent aberration in the nonpapillary RCC cell lines examined. This was expected because losses of 3p are one of the most common genetic aberra-

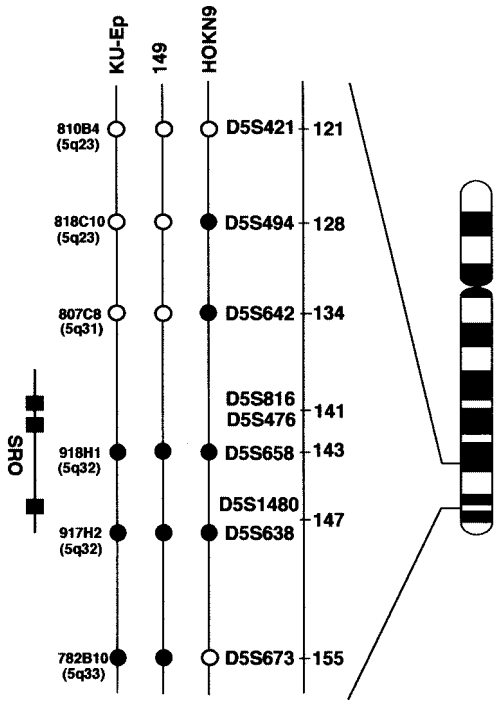

Fig. 4. Map of the region of chromosome 5q affected by CNAs detected by FISH in three nonpapillary RCC cell lines (KU-Ep, RCC149 and HOKN9), showing relative positions of YACs and microsatellite markers. Names of the YACs, and their cytogenetic locations according to the "Resources for Human Molecular Cytogenetics" and results confirmed by our FISH experiments, are indicated on the left; microsatellite markers and approximate genetic distances (in centimorgans) are on the right-hand side. Black circles denote increases in copy number, and open circles denote no detectable increases (see text). The smallest regions of overlap (SRO) among duplications observed in two distinct regions of 5q in primary nonpapillary RCCs (Bugert *et al.*, 1998) are indicated at the far left.

tions reported in primary nonpapillary RCCs studied by conventional cytogenetics.[3, 18] Loss of heterozygosity (LOH) analyses with restriction fragment length polymorphism (RFLP) markers have revealed that at least three putative tumor suppressor genes are present on this chromosomal arm, at 3p14, 3p21.3 and 3p25.[19] The locus at 3p25 contains the Von Hippel-Lindau (*VHL*) tumor suppressor gene, which is known to participate in development of sporadic renal tumors, especially nonpapillary RCCs.[20, 21]

Extensive cytogenetic studies have revealed that 5q-trisomies, (3;5) translocations leading to loss of 3p13-pter, and duplication of 5q22-qter are common features of nonpapillary RCCs.[22] This finding indicated that alterations of genes at chromosomes 3p and/or 5q are associated with the development of RCCs. According to RFLP analyses, allelic duplications of chromosome 5q22 occur in about 70% of primary nonpapillary RCCs, and arise also at 5q31.1 (D5S816–D5S1480; Fig. 4).[23, 24]

Our own results, derived from CGH and cytogenetic analyses in the series of nonpapillary RCC cell lines we examined, corroborate the conclusion that multiplication of 5q, particularly at 5q31-33, is a recurrent and nonrandom chromosomal aberration in this type of tumor. Furthermore, our FISH study using a number of YACs as probes revealed that the smallest region of overlap for multiplication of 5q lies within the genomic segment between D5S642 to D5S673, suggesting that gene(s) activated by a gene-dosage mechanism in this region may play important roles in nonpapillary renal-cell tumorigenesis. Indeed, numerous genes related to cell growth or proliferation, such as *PDGFRB*, *CSF1R*, *FGF1*, *IL9*, *CDC25* and *CDC25C*, have been mapped within this region (http://rmc-www.lbl.gov/CancerMap.html). *FGF1* is a critical factor for angiogenesis,[25] and over-expression of platelet-derived growth factor (PDGF) as well as its receptors is common in certain forms of human cancers such as glioblastoma.[26] We confirmed over-expression of *PDGFRA* and *PDGFRB* in the RCC19 cell line, which is amplified at 4q12, by western blotting (data not shown).

DNA losses on chromosomes 4q and 14q were more frequent in our series of nonpapillary RCC cell lines than in sporadic RCCs reported elsewhere.[27–29] Monosomy 14 has been proposed as a marker for increased malignancy in RCC[30]; other CGH studies have indicated frequent losses of chromosome 4q in RCCs with sarcomatoid transformation, i.e., the highest form of dedifferentiation in renal tissue.[31, 32] Our CGH results support a conclusion that chromosomes 4q and 14q may harbor tumor suppressors whose loss contributes to malignant progression of RCCs, because cell lines are generally established from highly aggressive neoplasms with extremely poor prognoses.

HLGs, even when detected infrequently, may highlight locations of dominant oncogenes involved in tumor progression. CGH analyses of primary nonpapillary RCCs have detected HLGs at 6p12-22, 7p21-22, and 11q22-23 in one case each.[27, 31] In the cell lines studied here, HLGs were found in eight different regions, each of them harboring a known growth-related gene (http://rmc-www.lbl.gov/CancerMap.html). By performing FISH experiments with YAC probes, we found that the HLG at 4q12 possibly involved amplifications of *c-kit* and *PDGFRA* in RCC19, a cell line established from a tumor with sarcomatoid transformation.

Papillary RCC cell lines did not show loss of 3p, but they did tend to gain whole or partial chromosomes such as chromosomes 7, 12 and 17. We confirmed these data by conventional Q-banding (data not shown) and noted their concordance with cytogenetic and CGH results obtained by other investigators.[3, 19, 33] SCC and TCC are unusual

forms of renal cancer, and renal SCC usually behaves aggressively. To our knowledge CGH analysis of these two types of tumor has not previously been reported.

The present study has underscored the genetic complexity and high average number of aberrations present in renal tumor cell lines. CGH analysis in this study also indicated the presence of genetic abnormalities in renal tumor that were not detected by conventional cytogenetic approaches. A comparison of our CGH data with published studies involving primary renal cancers indicates striking similarities of chromosomal gains and losses. However, ours is the first report to document frequent gains of 5q31-33 and high-level amplification of 4q12 in nonpapillary RCCs. The present CGH data should provide valuable information to guide the choice of individual cell lines for exploring target genes in regions of interest.

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