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# Allelic Loss at Chromosome 3p Characterizes Clear Cell Phenotype of Renal Cell Carcinoma[1]

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## ABSTRACT

**Incidence of the loss of heterozygosity on chromosome 3p was evaluated using 7 polymorphic probes in 35 Japanese patients with sporadic renal cell carcinoma (RCC). Overall frequency of the loss of heterozygosity on 3p was 53%, representing 16 of 30 informative cases. Examination of the relationship between histopathological phenotypes of RCC and incidence of the 3p loss revealed that the loss of heterozygosity in clear cell type tumors (75%, 12 of 16) was significantly ($P < 0.01$) more frequent than that in granular cell type tumors (14%, 1 of 7). In addition, three mixed cell type tumors, consisting predominantly of granular cell components, showed no loss of chromosome 3p loci. These findings may support the notion that the loss of heterozygosity on chromosome 3p is a nonrandom event in the tumorigenesis of sporadic RCC, and suggest that this type of chromosomal rearrangement is specific to the clear cell phenotype of RCC.**

## INTRODUCTION

Loss of genes at several specific chromosomal loci is implicated in the development of a variety of human and animal tumors (1), and the potential role of tumor suppressor genes has been an interesting subject of investigation regarding the genesis or progression of many human tumors (2, 3). In human RCCs,[3] previous cytogenetic studies demonstrated that the chromosomal rearrangement of the short arm of chromosome 3 occurred not only in hereditary RCC but also in the sporadic form of RCC (4–9). Recent RFLP analyses have provided additional evidence at the molecular level for loss of important loci on chromosome 3p in RCC (10–13). More recently, Shimizu *et al.* (14) demonstrated by the chromosome transfer method that the normal chromosome 3p could suppress or modulate the tumorigenicity of a human RCC cell line. These studies strongly suggest that a certain gene(s) located on chromosome 3p is responsible for the tumorigenesis of RCC.

In the present study, we examined 35 Japanese patients with sporadic RCC by the RFLP method with 7 polymorphic probes mapped to chromosome 3p to assess the incidence of chromosomal rearrangement of chromosome 3p in Japanese, with special attention to the relationship between the frequency of 3p loss and the histopathological phenotypes.

## MATERIALS AND METHODS

**Samples.** Thirty-five RCC patients were selected from patients with sporadic RCC treated at our hospital or at other community hospitals in Japan. None of the patients had undergone chemotherapy before nephrectomy. One patient had received preoperative immunotherapy with interferon-$\alpha$. Tumor tissues and intact portions of surgically removed specimens were snap-frozen after nephrectomy and stored at −70°C until DNA extraction. In addition, at least two portions of each tumor were fixed in 10% formalin for routine histological examination.

**Histological Examination.** Histological examination was performed on at least 2 portions of each tumor specimen. Tumors were classified according to the modified WHO classification (15); being classified as solid, alveolar, tubular, cystic, or papillary by histological configuration, and as clear, granular, mixed, pleomorphic, or spindle by cell type. The diagnosis of mixed cell type tumor was made in case that the tumor contained clear or granular cell components exceeding 20% of all tumor tissue examined.

**Polymorphic Probes.** The following 7 probes were used to analyze the loss of heterozygosity on chromosome 3p: p627 (16), pBH302 (17), pH3H2 (18), pHF12–32 (19), B67 (20), pMS1–37 (19, 21), and pEFD145.1 (22). Corresponding locus symbols, the restriction endonucleases used, the size and the frequency of polymorphic alleles, and their map locations are shown in Table 1 in accordance with the Tenth International Workshop on Human Gene Mapping (23).

**DNA Isolation and Southern Blot Transfer.** Tissue samples were homogenized mechanically and incubated in tissue lysis buffer (4 M urea, 200 mM NaCl, 0.5% sarcosyl, 10 mM EDTA, 100 mM Tris-HCl, 0.5 mg/ml proteinase K, pH 7.5) for 16 h at 50°C. After phenol/chloroform extractions and ethanol precipitation, DNA was dissolved in 10 mM Tris-HCl with 1 mM EDTA, pH 7.4. Each 10 $\mu$g of DNA was digested with 100–120 units of restriction endonucleases overnight under the appropriate reaction conditions. The digested DNA was electrophoresed on 0.8–1.0% agarose gels (Sigma, St. Louis, MO), and then transferred to nylon membranes (Biodyne A; Pall Biosupport, New York, NY).

**Hybridization.** The membranes were prehybridized in hybridization solution consisting of 50% deionized formamide, 5× Denhardt's solution (1× Denhardt's solution: 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 5× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.5% SDS, 50 mM sodium phosphate, and 250 $\mu$g/ml denatured sonicated salmon sperm DNA for 4–20 h at 42°C. To introduce radioactive nucleotide phosphates into DNA probes, the random primer method was performed with Mixed Primer Labeling System 1 (Clontech, CA). The unincorporated radioactive nucleotides were removed on a Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column. The membranes were then incubated in hybridization solution added with denatured radioactive probes at 42°C overnight with gentle agitation. After hybridization, the membranes were washed 3 times in 2× SSC, 0.5% SDS at room temperature for 5 min, and twice in 0.1× SSC, 0.5% SDS at 65°C for 15 min. Autoradiography was performed at −70°C for 1–7 days.

**The Determination of Loss of Heterozygosity.** Individuals whose normal kidney tissues were heterozygous on at least one locus were informative for detection of allelic loss. To quantitate the signal intensity of the polymorphic alleles, the hybridization signals were measured by scanning with a densitometer (LKB Ultroscan XL laser densitometer). The ratio of two polymorphic allele signals was calculated to correct for differences in DNA loading, and then the loss of heterozygosity was determined when the intensity of one allele signal of tumor tissue was reduced by more than 30% compared with the corresponding signal of normal kidney tissue. In addition, each densitometric determination was normalized for variations in amount of DNA between lanes using

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[3] The abbreviations used are: RCC, renal cell carcinoma; RFLP, restriction fragment length polymorphism; SSC, standard saline-citrate; SDS, sodium dodecyl sulfate.

Table 1 *Chromosome 3p loci tested for loss of heterozygosity*

| Locus[a] symbol | Probe | Enzyme | Alleles: Size (kilobases) | Alleles: Frequency | Map location |
|---|---|---|---|---|---|
| RAF1 | p627 | *Taq*I | 6.8/6.3 | 0.74/0.26 | 3p25 |
| | | *Bgl*I | 4.0/3.3 | 0.54/0.46 | |
| THRB(erbA$\beta$) | pBH302 | *Hind*III | 7.0/5.5 | 0.33/0.67 | 3p24.1-p22 |
| DNF15S2 | pH3H2 | *Hind*III | 2.3/2.0 | 0.46/0.54 | 3p21 |
| D3S2 | pHF12-32 | *Msp*I | 2.9/1.3, 1.6 | 0.83/0.17 | 3p21 |
| D3S4 | B67 | *Taq*I | 13/12 | 0.14/0.86 | 3pter-q21 |
| | | *Msp*I | 4.8/1.6 | 0.16/0.84 | |
| D3S3 | pMS1-37 | *Msp*I | 4.85/3.65 + 1.2 | 0.04/0.96 | 3p14 |
| D3S32 | pEFD145.1 | *Taq*I | 9.0/4.0 | | 3p |

[a] According to the Tenth International Workshop on Human Gene Mapping (23).

Table 2 *Clinical summary of informative patients*

| | No. of patients |
|---|---|
| All cases | 30 |
| Age[a] | 31–78 (mean 57.2) |
| Sex | |
| M | 17 |
| F | 13 |
| TNM stage | |
| pT2 | 22 |
| pT3 | 5 |
| pT4 | 3 |
| N0 | 28 |
| N+ | 2 |
| M0 | 26 |
| M1 | 4 |
| V0 | 24 |
| V+ | 6 |
| Robson's[b] stage | |
| I | 18 |
| II | 3 |
| III | 3 |
| IV | 6 |

[a] At diagnosis.
[b] According to Robson *et al.* (24).

the autoradiographic signal of a heterozygous locus of another chromosome and using the same membrane. In some cases, new sets of blots were made to confirm allelic losses.

## RESULTS

In 30 of 35 patients examined, DNA extracted from normal kidney tissues showed constitutional heterozygosity for at least 1 of 7 loci on chromosome 3p. In the other 5 patients (14%, 5 of 35), no information could be obtained for any locus examined, and constitutional heterozygosity for the D3S3 locus was not detected in any patient (data not shown). Recently, Dr. M. Yamada[4] observed that the frequency of heterozygosity for some polymorphic probes in Japanese patients was lower than that expected in Caucasians. It seems possible that the low frequency of heterozygosity for 3p probes, especially D3S3 and DNF15S2 probes, demonstrated in our study depends on the genetic characteristics of the Japanese. Of the informative 30 patients, 16 (53%) showed loss of heterozygosity on chromosome 3p. The clinical data from the 30 patients are summarized in Table 2. Since all specimens were obtained from operable patients, the spread of the primary tumor was confined within the renal capsule [Robson's stage I (24)] in over half of the patients.

Of the 30 informative patients, 16 tumors were histopathologically classified as the clear cell type. Among them, allelic loss at the 3p loci was found in 12 (75%) cases (Table 3; Fig. 1). Allelic loss at the 3p loci could not be demonstrated in any of the remaining 4 patients (patients 13–16) with clear cell RCC. However, the possibility of telomeric deletion cannot be excluded, since no information at RAF1, erbA$\beta$, or DNF15S2 loci was available in these 4 cases. On the other hand, allelic loss at the 3p loci was demonstrated in only 1 of 7 (14%) granular cell type RCCs (Table 4). This incidence was significantly lower than that in clear cell type RCCs (Fisher's exact test, $P < 0.01$). All 5 patients with mixed cell type tumor were informative at RAF1 or erbA$\beta$ locus (Table 4). Of these, 2 cases (patients 24 and 25) containing predominantly clear cell components showed loss of heterozygosity. In contrast, the other 3 cases (patients 26–28), consisting predominantly of granular cell components, retained heterozygosity at the 3p loci.

The comparative histopathological features of tumors and loss of chromosome 3p loci are summarized in Table 5. Three papillary type tumors (patients 20, 22, and 27) did not show any loss of the loci on chromosome 3p (Table 4). However, there was no correlation of the loss of heterozygosity at 3p with either architectural configuration or cytological grading.

Table 6 shows the incidence of 3p loss at each tumor stage. Although the number of high-stage tumors was small, no definite correlation could be observed between the tumor stages and the incidence of allelic loss at 3p.

In all patients examined, total allelic loss or new rearrangement bands were not observed at any locus.

## DISCUSSION

RCC is the most common malignant tumor of the kidney, and epidemiologically, this cancer has a relatively low incidence in the Japanese population (2 of every 100,000) compared with the highest incidence (8 of every 100,000) in Scandinavian countries (25). Of the 35 Japanese cases of sporadic RCC, loss of heterozygosity on chromosome 3p was detected in 16 of 30 informative cases (53%). The frequency in our series was slightly lower than those previously reported from other countries (10–13). However, the loss of heterozygosity was also demonstrated in over half of our Japanese cases, suggesting that the loss of heterozygosity at the loci on chromosome 3p is a nonrandom event in tumorigenesis of RCC.

RCC presents a variety of histopathological features both in architectural configuration and in cell type (15, 26). Typical RCCs are composed of clear or granular cells with pure or mixed arrangement; clear cells contain abundant lipid and glycogen, and granular cells contain numerous cytoplasmic organelles, especially mitochondria. However, it remains controversial whether these two cell phenotypes convey different malignant potential (27). In the present study, 12 of 16 (75%) clear cell RCCs exhibited loss of heterozygosity on chromosome 3p. In particular, 8 of these were informative at the 3 telomeric loci (RAF1, erbA$\beta$, or DNF15S2), all showing the loss of heterozygosity at these loci. In contrast, allelic loss on 3p was demonstrated in only 1 of 7 granular cell type tumors. The cytogenetic study by Carroll *et al.* (8) revealed that the rearrangement of chromosome 3 occurs in clear cell RCC at high frequency. More recently, Bergerheim *et al.* (13), using RFLP analysis, demonstrated that the loss of heterozygosity at 3p loci had occurred in 9 of 12 clear cell type tumors, whereas only 1 of 4 granular cell tumors showed loss of heterozygosity, but they did not refer to this difference. These observations suggest that the genetic events involved in tumorigenesis may be different between these 2 typical cell types of RCC. Kovacs *et al.* (28,

[4] M. Yamada, personal communication.

Table 3 *Loss of heterozygosity on chromosome 3p in clear cell type RCC*

| Patient no. | Histological[a] configuration | Cytological[b] grade | Locus symbol | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | RAF1[c] | erbAβ | DNF15S2 | D3S2 | D3S4[d] | D3S32 |
| 1 | CYS > ALV | 1 | —[e] | ◑ | — | — | Ⓓ | Ⓓ |
| 2 | ALV | 2 | ◑ | — | — | ◑ | — | ◑ |
| 3 | ALV > CYS | 1 | — | — | — | — | ◑ | — |
| 4 | ALV | 1 | ◑ | ◑ | — | — | ◑ | — |
| 5 | CYS > ALV | 1 | — | — | — | ◑ | — | ◑ |
| 6 | ALV | 1 | — | — | ◑ | — | — | ◑ |
| 7 | ALV | 2 | ◑ | — | — | — | — | — |
| 8 | ALV | 1 | ◑ | ◑ | — | — | ◑ | ◑ |
| 9 | ALV | 1 | — | — | — | ◑ | — | ◑ |
| 10 | ALV | 1 | — | — | — | — | ◑ | — |
| 11 | ALV | 1 | — | ◑ | — | ◑ | ◑ | — |
| 12 | ALV | 1 | ◑ | ◑ | — | ◑ | ◑ | ◑ |
| 13 | ALV | 1 | — | — | — | Ⓓ | — | — |
| 14 | ALV | 1 | — | — | — | — | Ⓓ | — |
| 15 | ALV | 1 | — | — | — | Ⓓ | Ⓓ | Ⓓ |
| 16 | ALV | 1 | — | — | — | — | Ⓓ | — |

[a] CYS, cystic; ALV, alveolar.
[b] According to Thoenes *et al.* (26).
[c] Allelic loss was tested by using 2 restriction endonucleases: *Taq*I and *Bgl*I.
[d] Allelic loss was tested by using 2 restriction endonucleases: *Msp*I and *Taq*I.
[e] —, No information; ◑, loss of heterozygosity; Ⓓ, retention of heterozygosity.

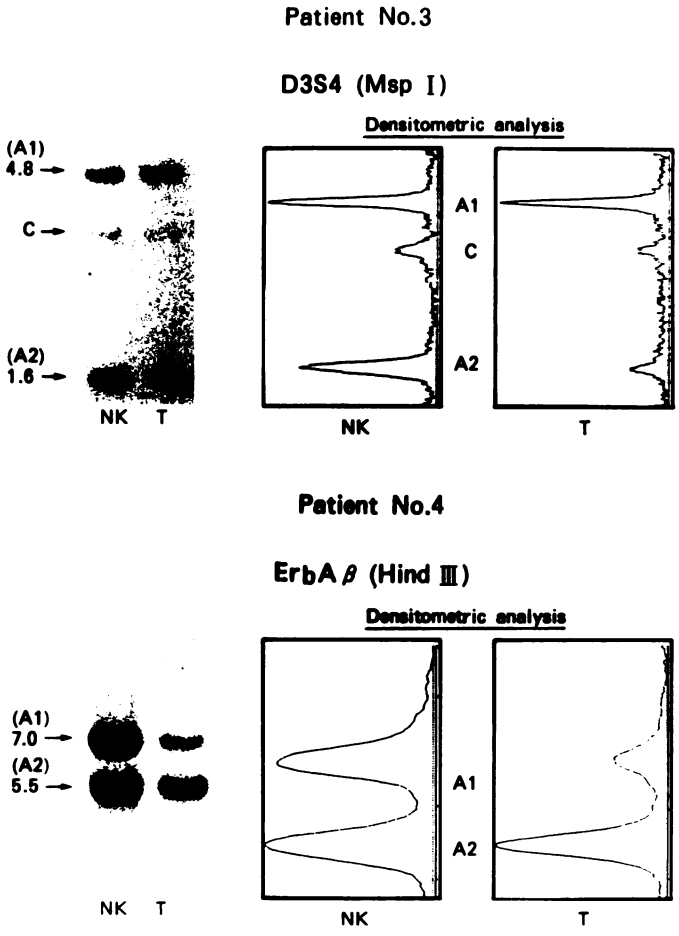


Fig. 1. Loss of heterozygosity on chromosome 3p in patients 3 and 4. In patient 3, the densitometric analysis reveals that the A2 allele of D3S4 in tumor tissue (*T*) decreases by 74% compared with normal kidney tissue (*NK*); *C*, constant band. In patient 4, the A1 allele of erbAβ in tumor tissue decreases by 64%.

29), employing both cytogenetic and RFLP analyses, reported the rearrangement of one of the chromosome 3 homologues in 81 of 85 nonpapillary type RCCs, whereas all of 11 papillary type tumors failed to show any abnormality on 3p. Similarly, 16 of 27 (60%) nonpapillary type tumors in our series showed the loss of heterozygosity on 3p, whereas none of the 3 papillary type tumors did. These findings also provide further evidence that the genetic events responsible for the genesis of RCC are different between the histological subtypes.

Kovacs *et al.* (28, 29) proposed that the loss of both alleles of tumor suppressor gene on 3p is a critical event in the development of RCCs regardless of their histological phenotypes. If this is true, it is possible that the submicroscopic alterations on 3p that cannot be detected with RFLP analysis might occur in granular cell and papillary subtypes of RCC, and that visible chromosomal alterations accompanied by some characteristic gene losses might be responsible for the histogenesis of clear cell or nonpapillary RCCs. However, additional studies are required to confirm the chromosomal alterations in RCC in relation to their phenotypic expression.

In the present series, 3 cases of mixed cell type tumor, which consisted predominantly of granular cell components, were identified. It is of interest that all of these tumors revealed retention of heterozygosity on 3p. This may be explained by 2 possibilities. (*a*) These tumors may be heterogeneous with regard to chromosome 3p rearrangement as well as to phenotypic appearance, therefore signal reduction could not be detected. (*b*) It is possible that loss of heterozygosity was absent in all components of these mixed type tumors. Kovacs *et al.* (11) claimed that genetic heterogeneity among RCC tumor cells was unlikely since the aberration of chromosome 3p was present in the entire clonal tumor cell population, favoring the latter interpretation. This possibility has also been alluded to by Linehan *et al.* (30), who examined the tumors from 3 RCC patients by RFLP analysis and revealed that nearly complete loss of partially reduced signal could be obtained after removal of contaminated lymphocytes by an immunological selection method, indicating the clonal deletion of chromosome 3p. According to these observations, it seems probable that loss of heterozygosity on chromosome 3p had not occurred in our mixed cell type RCCs.

There is agreement that RCC originates from the epithelium of proximal convoluted tubules (31, 32), but no definitive premalignant lesion for RCC has been identified. However, renal adenomas including specific types of oncocytoma possess close morphological similarities to RCCs and have been regarded as the candidates for premalignant lesion of RCC (33–

Table 4 *Loss of heterozygosity on chromosome 3p in non-clear cell type RCC*

| Patient no. | Cell[a] type | Histological configuration | Cytological grade | Locus symbol | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | RAF1 | erbAβ | DNF15S2 | D3S2 | D3S4 | D3S32 |
| 17 | GCC | ALV | 2 | — | — | — | — | — | ◑ |
| 18 | GCC | ALV | 2 | ◐ | ◐ | — | ◐ | — | ◐ |
| 19 | GCC | ALV | 2 | ◐ | — | ◐ | — | ◐ | ◐ |
| 20 | GCC | PAP > TUB | 1 | — | ◐ | — | ◐ | ◐ | ◐ |
| 21 | GCC | ALV | 1 | — | — | — | — | — | ◐ |
| 22 | GCC | PAP | 1 | — | — | — | — | — | ◐ |
| 23 | GCC | ALV | 1 | — | ◐ | — | ◐ | ◐ | ◐ |
| 24 | Mixed (C > G) | ALV | 2 | — | ◑ | — | — | ◑ | ◑ |
| 25 | Mixed (C > G) | ALV | 1 | ◑ | — | — | ◑ | ◐ | — |
| 26 | Mixed (C = G) | TUB | 1 | ◐ | — | — | ◐ | — | ◐ |
| 27 | Mixed (G > C) | PAP > ALV | 1 | ◐ | — | — | — | — | ◐ |
| 28 | Mixed (G > C) | ALV | 2 | ◐ | — | — | — | — | ◐ |
| 29 | PLM | SOL | 3 | ◑ | ◑ | — | ◐ | ◐ | — |
| 30 | SPN | SOL | 3 | ◐ | — | — | ◐ | — | ◐ |

[a] GCC, granular cell type; Mixed, mixed cell type; G, granular cell component; C, clear cell component; PLM, pleomorphic cell type; SPN, spindle cell type; ALV, alveolar; PAP, papillary; TUB, tubular; SOL, solid.

Table 5 *Histopathological features and loss of heterozygosity on chromosome 3p*

| | No. of cases with loss of heterozygosity/ no. of informative cases | (%) |
|---|---|---|
| All cases | 16/30 | (53) |
| Cell type[a] | | |
| Clear cell | 12/16 | (75) |
| Granular cell | 1/7 | (14) |
| Mixed cell | 2/5 | (40) |
| Other | 1/2 | (50) |
| Configuration[b] | | |
| Papillary | 0/3 | (0) |
| Nonpapillary | 16/27 | (60) |
| Cytological grade | | |
| 1 | 11/21 | (52) |
| 2 | 4/7 | (57) |
| 3 | 1/2 | (50) |

[a] Clear *versus* granular ($P < 0.01$).
[b] Dominant architectural configuration.

Table 6 *Tumor stages and loss of heterozygosity on chromosome 3p*

| | No. of cases with loss of heterozygosity/ no. of informative cases | (%) |
|---|---|---|
| All cases | 16/30 | (53) |
| TNM stage | | |
| pT2 | 13/22 | (59) |
| pT3 | 2/5 | (40) |
| pT4 | 1/3 | (33) |
| N0 | 15/28 | (54) |
| N+ | 1/2 | (50) |
| M0 | 13/26 | (50) |
| M+ | 3/4 | (75) |
| V0 | 13/24 | (54) |
| V+ | 3/6 | (50) |
| Robson's stage | | |
| I | 10/18 | (56) |
| II | 1/3 | (33) |
| III | 1/3 | (33) |
| IV | 4/6 | (67) |

35). It is well known that tumor cells composing renal oncocytoma are sometimes difficult to distinguish from granular cells of RCC (36, 37). In addition, renal cortical adenoma, which is not infrequently found at autopsy, has a close resemblance to papillary type RCC, and therefore has been regarded as a silent form of RCC by several investigators (38, 39). According to the previous cytogenetic studies (35, 40, 41), all but one case of renal oncocytoma manifested no visible rearrangement of chromosome 3. Also, in recent RFLP studies by Kovacs *et al.* (42) and Brauch *et al.* (43), renal oncocytomas were reported to retain the alleles of chromosome 3p. The absence of rearrangement on chromosome 3 was demonstrated in 3 large renal adenomas as well as by cytogenetic investigation by Cin *et al.* (44). In view of the lack of, or low incidence of chromosomal rearrangement on chromosome 3 in both renal adenomas and granular cell and papillary type RCCs, the possibility that renal adenomas are interpreted as premalignant lesions with potential to develop towards granular cell or papillary type RCC cannot be excluded. However, more detailed molecular analysis on a large number of renal tumors is necessary, and it remains to be elucidated whether or not renal adenomas are histogenetically related to RCCs.

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