Investigation of recurrent deletion loci specific to conventional renal cell carcinoma by comparative allelotyping in major epithelial carcinomas

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ABSTRACT

Objective: Loss of heterozygosity (LOH) studies were undertaken to investigate the consistently deleted loci/? tumor suppressor gene loci (TSG) on 3p in conventional renal cell carcinoma (cRCC).

Materials and Methods: LOH studies were performed by polymerase chain reaction (PCR) using 15 micro satellite markers mapped in region 3p12-p26 on 40 paired cRCC tumors and normal kidney at Stages I-IV. Simultaneously, fluorescent in-situ hybridization (FISH) studies were performed to investigate the allelic deletion of fragile histidine triad (*FHIT*).

Results: Our studies revealed three affected regions; 3p12.2-p14.1, 3p14.2-p21.1, and 3p24.2-p26.1 with differential frequencies in Group I (Stage I and II) and Group II (Stage III and IV). Incidence for D3S1234 (*FHIT* locus) and D3S2454 (3p13) was 75% and 83% in Group I and II, respectively. Comparative allelotyping in epithelial malignancies like lung, bladder, and breast tumors revealed LOH (frequency 14–20%) only in breast tumors for D3S2406, D3S1766 (distal to *FHIT*), and D3S1560 (distal to *VHL*, Von-Hippal Lindau). FISH using *FHIT* gene probe revealed deletions in cRCC (88%), breast (30%), and lung tumors (10%) with no deletions in bladder tumors and leukemias, signifying the importance of *FHIT* in the pathogenesis of tumors of epithelial origin.

Conclusion: Our findings suggested *FHIT* deletion as an early and *VHL* deletion as an early and/or late event in cRCC. Additionally, studies also disclosed the recurrent deletions of flanking loci to *FHIT* and *VHL* in cRCC. The dilemma of interstitial or continuous deletion on 3p needs to be resolved by implementation of latest sensitive molecular techniques that would further help to narrow down search for TSG loci specific to cRCC, other than *VHL* and *FHIT*.

Key words: 3p, Conventional renal cell carcinoma, comparative allelotyping, *fragile histidine triad*, interstitial deletion, *von hippal lindau*

INTRODUCTION

A large number of sporadic conventional renal cell carcinomas (cRCC) are frequently associated with the loss of heterozygosity (LOH) on the short arm of chromosome 3, i.e. 3p.[1-7] In cRCC, the von Hippal Lindau (*VHL*) gene at locus 3p25.3 is believed to be a primary targeted tumor suppressor gene (TSG) and is inactivated by various mechanisms such as LOH, point mutations and epigenetic mechanisms such as hypermethylation.[8] The fragile histidine triad (*FHIT*) gene at locus 3p14.2 is one of the universal TSGs; however, the role of *FHIT* in the pathogenesis of cRCC is still not clear. Review of literature of LOH on 3p in cRCC by various groups has shown differential regions on 3p that were targets of deletions by LOH analysis.[3-7] Braga *et al*[4,9] have studied the entire region of 3p by micro satellite markers and found LUCA (Lung cancer region) (centromeric) and AP20 (Alu-PCR clone 20) (telomeric) at locus 3p21.31 as hotspot loci of deletion in cRCC. On the other hand, Velickovic *et al*[3] studied only the *VHL* gene at 3p25.3 and the *FHIT* gene at 3p14.2 using a set of micro satellite markers that flanked these genes and found the highest LOH at the *FHIT* locus than the *VHL* locus in low grade tumors. Except for Velickovic *et al*,[3] statistical analysis of the association of LOH loci on 3p with clinico-pathological parameters such as tumor grade and stage is unknown. Therefore, the pathogenetic role of deleted loci

in the biology of the disease is still not clear. Hence, we performed deletion mapping studies by LOH on 3p12-p26 in our series of cRCC patients at various stages and grades of disease with an aim to investigate the consistently deleted locus/loci and their specificity in cRCC by comparative allelotyping studies in cRCC and other major epithelial carcinomas (MEC) such as breast, lung, and bladder tumors.

MATERIALS AND METHODS

We collected 70 tumor specimens of RCC in a prospective manner from patients who underwent surgical resection of tumors by radical nephrectomy during the period between 2003 and 2006. Simultaneously, normal kidney specimens were also surgically resected along with the tumor of the same patient. Informed consent of all patients enrolled in this study was procured at all collections as per the ethical guidelines of the hospital. Staging of the disease was performed by the surgeon according to the American Joint Committee in Cancer (AJCC) Tumor Node Metastasis (TNM) staging (1997), while tumor morphological grading was performed by the pathologist (Dr SB Desai). Overall studies were carried out on 40 cRCC patients only after histopathological verification. The stage and grade wise distribution of these patients is as given below:

Stage I: 6   Grade I: 1
Stage II: 12   Grade II: 18
Stage III: 19   Grade III: 19
Stage IV: 3   Grade IV: 2

We categorized the patients into two groups: Group I comprising of patients of Stage I and II, and Group II comprising of patients in Stage III and IV for statistical analysis due to inadequate number of patients in Stage I and IV.[10] Similar grouping was done for statistical analysis with tumor grade.

The tumor tissues were minced and digested with Collagenase Type II S (Sigma Aldrich) and the single tumor cells obtained after digestion were grown *in vitro* to obtain cultures containing tumor cells free of fibroblasts. These cultured tumor epithelial cells subjected to DNA extraction for LOH studies by Polymerase Chain Reaction (PCR). Simultaneously, genomic DNA was also extracted from the normal kidney tissues by the phenol-chloroform method and subjected for PCR analysis. A part of the tumor was also used to prepare tumor imprints in all 40 tumors of cRCC for fluorescent in-situ hybridization (FISH) studies. Comparative allelotyping was also performed on extracted DNA of 15 paired normal tumor tissues of three MEC's such as breast, lung, and bladder. Tumor imprints of all these tumors were also prepared for FISH studies. Collection of these non-renal tissues was also approved by the ethical committee and the specimens were obtained with the help of a pathologist after histopathological investigations.

Micro satellite Analysis

Deletion mapping studies by LOH were carried out using 15 polymorphic micro satellite markers, which cover the entire region of 3p from 3p12-p26 [Figure 1]. The primer sequences, hetero/polymorphic nature of alleles, allele length and their respective loci on 3p were confirmed from Genome database (GDB) (http://*www.gdb.org*), location database (LDB) (*http://www.cedar.genetics.soton.ac.uk/pub/chrom3/gmap*) and co-operative human linkage centre (CHLC) (*http://www.chlc.org*) databases and Genome directory published by Gemmill *et al.*[11] The primers were custom made from Bioserve Biotech, Hyderabad, India. Each pair of micro satellite primers used in the current study were used to amplify corresponding complementary sequence defined within the flanking primers on the respective target sequence on the template. The length of each of the micro satellite primers ranged from 20–25 mer. Because of their repetitive binding patterns, Hot-start PCR was carried out to allow primer-specific binding to the unique target sequence on the template strand. Hot-Start-PCR was performed on thermal cycler (Techne Progene) in a 25 µls reaction by using 1× PCR buffer (Finnzymes, Finland), 1.5–3 mM $MgCl_2$ (Finnzymes, Finland), 200 µM each deoxyribonucleoside triphosphates (GIBCO, Invitrogen), 10 picomole/µl of both forward and reverse primer, and 50 ng of template genomic DNA. Hot-start PCR program for all micro satellite primers used was set up as follows:

Initial Denaturation: 94°C for 5 minutes – 1 cycle (Addition of Taq polymerase)
Denaturation: 94°C for 20–45 seconds,
Annealing: 42–56°C for 40 seconds–1.15 minutes,
Extension: 72°C for 15–20 seconds (All 3 segments for 32–35 cycles).
Final extension: 72°C for 5 minutes– 1 cycle.

Taq DNA polymerase was added after initial denaturation at 85°C. Five microliters of the Hot-start PCR products were run on 12% or 15% polyacrylamide gel using 1X TBE buffer and stained with 0.2% silver nitrate using protocol of Caetano-Anolles *et al.*[12] LOH was determined by comparing the intensities of the bands of the normal and tumor DNA. Thus, three band patterns could be obtained, LOH, Allelic imbalance (AI), (LOH mosaicism based on band intensity of alleles in both normal and tumor), and Micro satellite instability (MSI) by comparing the bands of tumor DNA with the corresponding band of the normal DNA. Aberrant LOH patterns for all specimens were repeated three times and confirmed by two independent experts.

Fluorescent in-situ hybridization (*FISH*) studies using fragile histidine triad (*FHIT*) bacterial artificial chromosome (BAC) genomic clone in cRCC and other malignancies:

FISH was performed on tumor imprints of all 40 cRCCs, 15 samples each of lung, bladder, and breast tumors and 10 samples each of mesenchymal bone marrow specimens of

leukemias such as acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) to evaluate the deletion status of *FHIT* gene. The *FHIT* BAC clone (RP11-170K19) was provided to us as a gift by Dr. Reiner Seibert, Germany. This clone is around 175kb and is mapped at locus 3p14.2. The *FHIT* gene probe was prepared In-House in our laboratory by growing the *FHIT* BAC clone in Luria-Bertanii (LB) medium, extracting the BAC DNA and labeling with flourochromes such as Cy-3/FITC-11-dUTP by nick translation method. In brief, the FISH probe labeling was performed as follows: 1 μg of BAC DNA was mixed with 5 μls of 10× reaction buffer (1×), 0.5 mM dNTP's of dATP, dCTP, and dGTP each (GIBCO, Invitrogen), β-Mercapto-ethanol (0.5 M), DNAse I (working stock: 0.00023 units), DNA polymerase I (Roche Diagnostics) (10 U/ml) and 0.5 μl (0.5 nmole) of Cy3-dUTP/ Flourescein-dUTP in labeling reaction mix of total volume 50 μls. After incubation at 15°C for 1–2 hours, labeled-DNA was precipitated with 3M Na acetate and 100% chilled absolute alcohol at –80C for 2 hours. The DNA precipitate was dried and dissolved in FISH hybridization buffer. The flourochrome-labeled probe was then used directly for FISH. The protocol followed for *FHIT* BAC probe FISH was as per our laboratory standardized protocols.[13,14] For FISH, the tumor imprint slides were passed through a series of alcohol grades. The probe mixture of volume 9 μl was made by mixing 2 μl labeled probe with 7 μl hybridization buffer. Denaturation of target DNA in interphase and metaphase cells affixed on slides was carried out by co-denaturing the slides along with the probe mixture at 73°C for 5 minutes followed by overnight hybridization on Thermobrite (Vysis Inc.) at 37°C overnight. Next day, the slides were washed at 72ºC in 0.4× Sodium chloride, sodium citrate and Nonidet 40 (SSCN) for 2 minutes, followed by wash in 2× SSCN at room temperature for 1 minute. The slides were counterstained and mounted in DAPI/Antifade (Vysis, Inc.). Signals were observed under epiflourescent Axioplan (Carl Zeiss, Germany) microscope using triple band pass filter. A total of minimum 200 cells were scored in each specimen. Simultaneously, FISH studies were also performed on 10 normal control peripheral blood specimens to evaluate the false positivity, sensitivity and locus specificity of the *FHIT* BAC probe.

Statistical analysis

Comparison of the LOH frequencies at all micro satellite loci on region 3p12-p26 was made with clinico-pathological parameters such as tumor stage and morphological grade to explore the significance of recurrent deleted loci. For this, Chi-Square analysis was applied. A two-sided P value of less than 0.05 was considered to be statistically significant. Statistical analysis was performed using the Special Performance System Software (SPSS) software.

RESULTS

Analyses of deletion mapping studies on 40 paired normal-tumor specimens of cRCC are depicted in Figures 1, 2, and 3. For data interpretation, we divided 3p12-p26 into four regions. All the markers in regions R1, R2, R3, and R4 are depicted in ascending order of their mapping.

Region 1: 3p12.2-3p14.1 (D3S2406, D3S2454, D3S1285).

Region 2: 3p14.2-p21.1 (D3S1480, D3S1481, D3S1300, D3S1234, and D3S1766). Except for marker D3S1766, the remaining four markers were mapped within exons 3 to 6 of the *FHIT* gene at locus 3p14.2.

Region 3: 3p21.2-p24.2 [D3S1568 (LUCA), D3S2409, D3S2420, and D3S1298 (AP20)].

Region 4: 3p24.2-p26 (D3S1597, D3S1317 - *VHL* gene locus, and D3S1560).

We found LOH in 3 regions, R1, R2, and R4 on 3p [Figure 3].

The region R1, 3p12.2-p14.1 of LOH near the pericentromeric region flanked by three markers, D3S2406, D3S2454, and D3S1285 revealed LOH frequency of 40%, 30%, and 15%, respectively.

In region R2, 3p14.2-p21.1, LOH at three of the five markers, D3S1300, D3S1234 and D3S1766, was 18%, 20% and 60% respectively. Two of the three markers, D3S1300 and D3S1234, mapped within the intron 5 of the *FHIT* gene revealed a low LOH. The locus 3p21.1 flanked by marker D3S1766 revealed comparatively higher LOH (60%). The other two proximal markers D3S1480 and D3S1481 of R2 close to R1 region, revealed retention of heterozygosity (ROH) [Figure 3].

The region R4, 3p24.2-p26 flanked by three markers, D3S1597, D3S1317 – *VHL* gene locus and D3S1560 revealed LOH frequency of 30%. Of these three markers, D3S1317 is mapped very closely to *VHL*, whereas marker D3S1560 is mapped 700kb distal to the *VHL* gene.[15]

In the region R3, 3p21.2-p24.2, markers D3S1568, D3S2409, D3S2420, and D3S1298 revealed comparatively low LOH frequency.

The frequency of MSI for all markers on 3p12-p26 was low (3–13%), except marker D3S1766 at 3p21.1, which had a higher MSI frequency (33%) in addition to a higher LOH rate (60%).

Among the LOH positive cases, more than 40% belonged to Group I. The incidence for intragenic *FHIT* gene markers, D3S1300 and D3S1234 and an extragenic marker D3S1766 in region R2 was 30%, 75%, and 40%, respectively in Group I patients. LOH frequency was significantly higher for marker D3S1234 in Group I in comparison to Group II
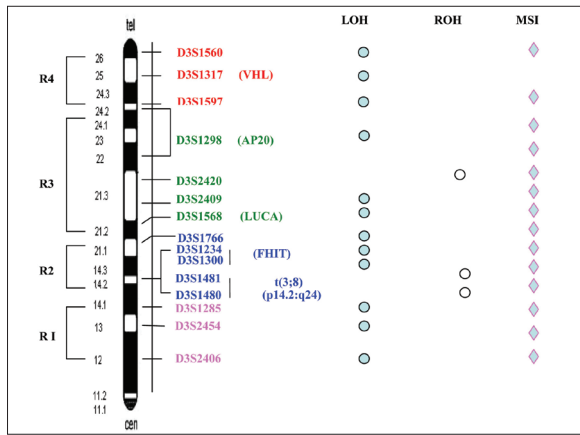
Figure 1: Chromosome 3p ideogram showing loss of heterozygosity (LOH), retention of heterozygosity (ROH), and micro satellite instability (MSI) for micro satellite markers mapped on 3p according to location database (LDB) in cRCC.
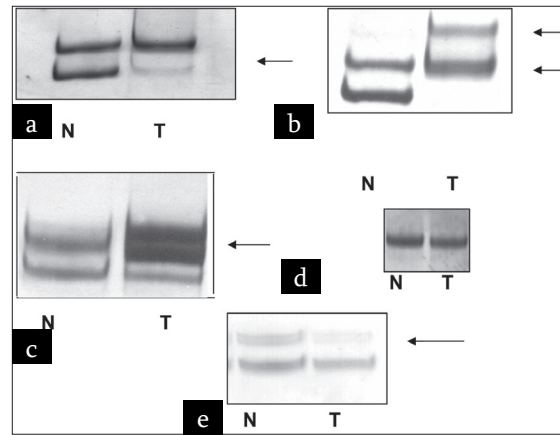

Figure 2: (a) LOH at locus 3p12.2 for micro satellite marker D3S2406 in cRCC. (b) MSI in both alleles at locus 3p21.1 for marker D3S1766 in cRCC. (c) Heterozygous MSI at locus 3p14.1-p14.2 for marker D3S1285 in cRCC. (d) Homozygous allelic pattern at locus 3p21.3 for micro satellite marker D3S2409 in cRCC. (e) LOH at locus 3p21.1 for marker D3S1766 in breast carcinoma.
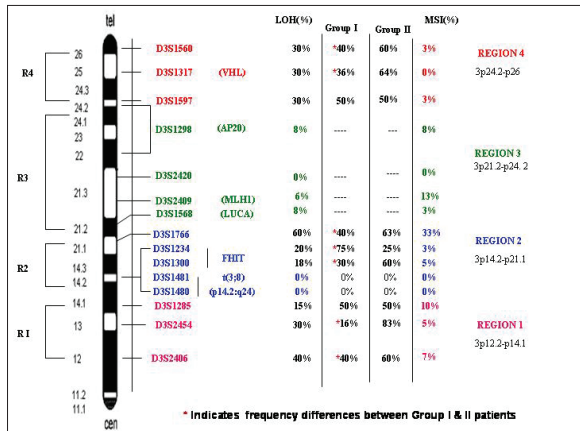**N:** Normal DNA T: Tumor DNAL

Figure 3: Chromosome 3p ideogram showing loci of LOH and MSI in defined regions R1, R2, R3, and R4 in cRCC patients. It also indicates differential LOH frequencies in Group I (Stage I and II) and Group II (Stage III and IV).

Figure 4: Chromosome 3p ideogram showing common LOH loci in defined regions R1, R2, R3, and R4 in cRCC and breast

patients (P < 0.013). The comparative incidence for *VHL* gene marker, D3S1317 and flanking markers, D3S1597 and D3S1560 in region R4 was 50%, 36%, and 40%, respectively and was comparatively higher in Group II patients [Figure 3]. Besides *FHIT* and *VHL* loci, the LOH frequency of marker D3S2454 at locus 3p13 also showed a significantly higher LOH in Group II (83%) than Group I (16%). Except for *FHIT* marker (D3S1234), LOH rates for most of the micro satellite loci were almost double for most of the markers in Group II as compared to Group I. LOH at the *FHIT* locus did not show any significant correlation with morphological grade of tumors in both groups of patients (P = not significant).

LOH studies of the affected LOH loci in other carcinomas:
Comparative allelotyping studies of the affected LOH loci of cRCC in MEC's such as breast, lung, and bladder carcinomas revealed ROH in lung and bladder carcinomas (data not shown). Except for low LOH frequency at markers D3S2406 (20%), D3S1766 (14%), and D3S1560 (20%) at loci 3p12.2, 3p21.1, and 3p25-p26 in Regions R1, R2, and R4,
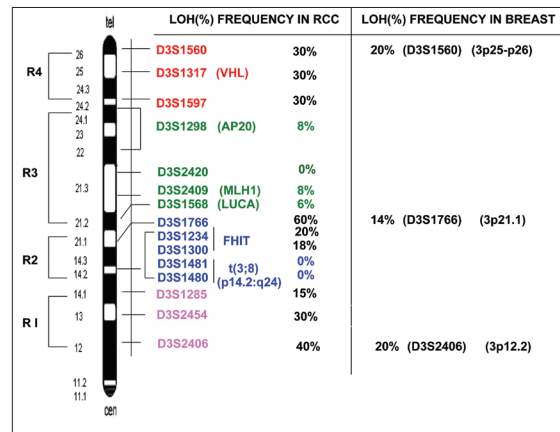
respectively, rest of the affected LOH loci of cRCC revealed ROH in breast carcinomas [Figure 4].

FISH analysis of *FHIT* gene on all 40 tumor imprints of cRCC revealed monoallelic deletions in 35 tumors (88%) with almost equal incidence in Group I and II patients. FISH analysis of *FHIT* gene in MEC's revealed allelic deletions only in 10% of breast and 30% of lung tumors. (data not shown). None of the leukemias revealed allelic deletions of the *FHIT* gene. The *FHIT* gene probe was initially tested on 10 peripheral blood specimens of normal controls and revealed 5% monoallelic loss. We considered an upper limit of ≥10% as positive in tumor specimens.

DISCUSSION AND CONCLUSION

Deletion mapping on 3p12–p26 revealed three consistently affected LOH regions; R1-3p12.2–p14.1, R2-3p14.2–p21.1 and R4-3p24.2–p26. The region R3, 3p21.2–p24.2 revealed a

comparatively low LOH frequency. Perhaps it could be due to homozygous allelic pattern for two markers D3S2409 and D3S2420 in 50–60% of patients. Braga et al[4] in their studies revealed 3p21.31 region (between LUCA and AP20 region) to be a hot spot LOH region. Maestro et al[16] in their study in cRCC revealed higher LOH frequency at 3p21.2-p21.3 (D3S1228) in comparison to 3p14.1-p14.2 (D3S1029) and 3p25 (D3S1038). Alimov et al[17] in their combined LOH and CGH analysis found regions 3p21.2 and 3p21.3-p22 along with 3p13-p14 and 3p25-p26 as regions of frequent LOH. Stage-wise analysis revealed that LOH at 3p14.2 was significantly higher in Group I patients than any other affected LOH loci on 3p, suggesting that LOH at FHIT locus may be an early event in cRCC and may have a potential pathogenetic role in cRCC. FISH analysis with FHIT gene probe in cRCC also supported LOH results. Further, findings of FHIT allelic deletions only in breast and lung tumors suggest that FHIT is involved in the tumorigenesis of cancers of epithelial origin rather than mesenchyme origin.

The pathogenetic role of FHIT in cRCC is still unclear because biallelic inactivation of FHIT (point mutations and LOH) as in other classical TSG's has not yet been identified.[5,18-20] FHIT mutation analyses have demonstrated LOH and hypermethylation as a common mutagenic event in carcinomas. Functional analysis of the FHIT gene has been only based on the expression profiles in various epithelial carcinomas including kidney tumors. LOH and reduced/altered expression of FHIT gene due to allelic deletions observed in lung, esophagus, head and neck, gastric, breast and cervical carcinomas have been associated with poor prognosis.[21] Various immunohistochemical studies in cRCC cell lines have shown that FHIT is either down-regulated or absent and are associated with Grade I tumors and early clinical stage and absence of FHIT expression was associated with progression of tumors in grade G2/G3.[22,23] Our studies in support with that reported by Velickovic et al[3] suggests that FHIT gene inactivation by itself may be a primary tumorigenic event and may require the deletions of other TSG loci on 3p as initiating events which are preceded by deletions of FHIT gene.

A two-time higher frequency of LOH at the other affected loci on 3p in Group II patients further suggests genetic instability of tumors during disease progression and increased susceptibility to deletions with higher frequencies, which further reflects the role of these loci in both disease development and progression as well.

The VHL TSG has been known to be frequently inactivated by LOH, point mutations or methylation in cRCC.[8] In our study, we found comparatively low LOH frequency (30%) than reported series.[3,6,17] The deletions of the VHL gene along with flanking loci, D3S1597 and D3S1560 in region 3p24.2-p26 is suggestive of long terminal deletions

in cRCC. LOH at the VHL locus (D3S1317) and flanking loci (D3S1560) was higher in Group II than in Group I patients, suggesting that deletion of VHL and flanking genes occur independently of FHIT during the later stages of development and/or progression of cRCC.

The occurrence of LOH at three loci on 3p in breast tumors [3p25-p26 (D3S1560), 3p21.1 (D3S1766) and 3p12.2 (D3S2406)] in our comparative allelotyping studies suggests the presence of common TSG's loci that are probably frequently deleted in both cRCC and breast tumors or there could be multiple genes in these loci which are differentially deleted and participate in formation of histologically different neoplasms. The findings of LOH at regions distal to VHL gene (D3S1560) in breast tumors suggest the involvement of TSG loci flanking VHL gene in breast tumorigenesis. The absence of VHL gene deletions in lung and bladder tumors further suggest specificity and role of VHL gene only in cRCC. The VHL gene studies in Indian population are nil in literature and our findings of VHL-LOH supports the reported literature that VHL gets inactivated by deletions other than mutations and methylation, thereby reflecting the role of VHL as a candidate TSG in the development and/or progression of cRCC and probably occurs independent of FHIT LOH.

Although FHIT and VHL deletion loci were common in reported and our series, the LOH pattern of other affected loci of discontinuous deletions was different in their and our series.[4-7,17] Additionally, our studies disclosed recurrent deletions of flanking loci to FHIT and VHL in our series of cRCC. The LOH of flanking loci of VHL (3p24.2-p26) is suggestive of long terminal deletion of 3p24.2-p26 is unique finding in our series.

Homozygous allelic patterns observed in two micro satellite markers in the region 3p21.2-p24.2 suggest the limitations of PCR to detect deletion for homozygous alleles. The dilemma of differential patterns of deletion needs to be resolved by the implementation other sensitive techniques such as real-time quantitative PCR, CGH array, and BAC Array-FISH, which would allow us to validate targeted LOH loci on 3p and to narrow down our hunt for hot spot loci on 3p to specific chromosomal sub bands. These studies in future would serve as a platform for positional cloning strategies to identify many putative or candidate RCC-related TSG's on 3p other than VHL and FHIT, which additionally contribute in the pathogenesis of cRCC.

ACKNOWLEDGMENTS

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