Absence or Reduction of Fhit Expression in Most Clear Cell Renal Carcinomas

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Abstract

The Fhit gene at human chromosome region 3p14.2 straddles the common fragile site, FRA3B, and numerous homozygous deletions in cancer cell lines and primary tumors. Also, the 3p14.2 chromosome breakpoint of the familial clear cell kidney carcinoma-associated translocation, t(3;8)(p14.2;q24), disrupts one Fhit allele between exons 3 and 4, fulfilling one criterion for a familial tumor suppressor gene: that one allele is constitutionally inactivated. Because the Fhit gene sustains biallelic intragenic deletions rather than mutations, there has not been evidence that the Fhit gene frequently plays a role in kidney cancer, although replacement of Fhit expression in a Fhit-negative renal carcinoma cell line suppressed tumor growth in nude mice. We have now assessed 41 clear cell renal carcinomas for expression of Fhit by immunohistochemistry. Normal renal tubule epithelial cells express Fhit uniformly and strongly, whereas 51% of the tumors are completely negative, 34% of tumors show a mixture of positive and negative cells, and 14% are uniformly positive, although usually less strongly positive than the normal epithelial cells. Most interestingly, there was a correlation between complete absence of Fhit and the G1 morphological grade and early clinical stage. Morphological grades G2 and G3 exhibited a mixture of positive and negative cells with a tendency for a higher fraction of negative cells in G3. Fhit inactivation is likely to be an early event in G1 tumors and may be associated with progression in G2 and G3 tumors.

Introduction

The short arm of chromosome 3 (3p) is altered by deletions or translocations in clear cell RCCs.2 Cytogenetic and molecular genetic studies have shown that several 3p regions are targets of allelic loss on 3p in clear cell tumors (1–3). The familial von Hippel Lindau disease locus (VHL), which predisposes to a variety of benign and malignant tumors, including clear cell RCCs, was mapped to 3p25, and the VHL gene was isolated, characterized, and shown to be inactivated not only in the VHL familial kidney cancers but in a large fraction of sporadic clear cell RCCs (4–7). In parallel, numerous studies of LOH of genetic loci spanning 3p refined and narrowed the different regions of 3p that were specifically targeted by allelic loss, and most RCCs exhibited loss of more than one 3p locus (8–10). Thus, individual RCCs had usually lost one or more of a combination of loci at 3p25 (VHL), 3p21.3, 3p14, and, possibly, 3p12. A locus at 3p14.2 had been considered a possible RCC tumor suppressor locus because of the chromosome translocation t(3;8)(p14.2;q24), which was associated with hereditary RCC in a large three-generation family (11, 12). A defined region at 3p14.2, encompassing the t(3;8) translocation breakpoint, was involved in LOH in more than 80% of sporadic RCCs (9), although it has been argued that the t(3;8) translocation, in which some of the familial tumors resulted in loss of the derivative 8 [der 8(8pter→8q24::3p14.2→3pter)] chromosome, might be a mechanism for loss of one VHL allele; that is, the real target of loss in the familial RCCs may be the VHL gene at 3p25 rather than a 3p14.2 tumor suppressor gene (5). Cytogenetic and LOH studies of sporadic RCCs have suggested to some observers that most sporadic RCCs do not exhibit interstitial deletions but rather suffer terminal loss of various 3p segments due to chromosome translocations with various breakpoints in the centromic half of 3p (3p11–3p14; Ref. 13). Most studies, however, do report apparent interstitial loss of 3p regions at p14, p21.3, or p25, and several studies have attempted to correlate losses at specific 3p regions with clinical parameters. Hadaczek et al. (10) did not observe such a correlation, whereas van den Berg et al. (14) concluded that allelic loss at 3p21 was a prerequisite for malignant development.

Materials and Methods

Tissues and Cell Lines. Samples were obtained from patients who underwent kidney resection because of renal cancer (Department of Urology, Medical Academy, Szczecin, Poland). Both normal and tumor tissues were fixed in 10% buffered formalin for 24 h and then embedded in paraffin. The study group comprised 41 cases of clear cell RCCs with variable morphological grades and clinical stages. For clinicopathological parameters, see Table 1. Many cases showed heterogeneity as to morphological grading. In such cases we marked them as G1/G2, which meant that the majority of cells had G1 features but there were also areas where G2 cells were present. RCC8, RC12, RC17, RC48, and RC49 RCC cell lines were obtained from Dr. Edmund Lattime (Jefferson Medical College); Fhit alterations in RC48 and RC49 were described previously (18). Other RCC cell lines were obtained from American Type Culture Collection; ACHN, CaKil, CaKilII, A498, A704, and RC cell lines were maintained in MEM with 10% fetal bovine serum.
Table 1  Fhit expression in clear cell RCCs

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a Mean age at diagnosis, 59 years.

b According to the criteria of Fuhrman et al. (25).

c According to the modified (27) criteria of Robson et al. (26).

d Immunoreaction intensity was marked as follows: (—), negative; (+), pale positive; (++, moderately positive; and (++), strongly positive. (—/+) means that the case showed heterogenous Fhit staining with a majority (>50%) of negative cells, whereas (+/-) depicts a heterogenous case with a majority (>50%) of positive cells.

Fresh normal kidney tissue and matched kidney carcinoma tissue was obtained from the KCI tumor bank, minced, and placed in tissue culture in RPMI with 10% fetal bovine serum. Most normal samples grew well in culture for two or three subcultures; ~50% of the carcinomas grew in culture for a few passages, and a few became established lines. DNA was prepared from fresh tissue and cultured cells; RNA was usually isolated from the cultured cells.

DNA and RNA Analyses. Methods of isolation of DNA and total RNA from cell lines and tissues have been described (18). Southern blot analysis of kidney cell DNAs for detection of alterations in the FHIT locus was performed as described previously, as were the PCR and nested RT-PCR amplification procedures for evaluation of integrity of FHIT exons and transcripts (18). For Northern blot analysis, 3 μg of poly(A)+ RNA for each cell line were electrophoresed in 0.8% agarose in a borate buffer containing formaldehyde, transferred to HybondN* membrane (Amersham, Arlington Heights, IL) using standard procedures, and hybridized to a FHIT cDNA probe.

Immunohistochemistry. Routine deparaffinization of all sections mounted on the silan coated slides was carried out according to standard procedures followed by rehydration through an ethanol series. The slides were immersed in citrate buffer (pH 6.0) and heated in a microwave oven (twice for 4 min). The sections were then incubated (1 h at 37°C) with the primary antibody, a polyclonal rabbit immunoglobulin against glutathione S-transferase-Fhit protein (Amersham, Arlington Heights, IL), followed by biotinylated antirabbit antibody and streptavidin-biotin-peroxidase (Histostain-SP kit, Zymed Laboratories, San Francisco, CA). Treated slides were washed, lightly counterstained with hematoxylin, and coverslipped prior to evaluation. When ever possible, we analyzed sections containing the border between tumor and normal tissue to have an internal positive control for each case.

Statistical Analysis. For analysis of statistical significance of correlations between clinicopathological parameters and Fhit expression, we used the Fisher exact test with a two-tailed P.

Evaluation of the FHIT Gene and Transcript in Kidney Cells. We and others have previously shown that one FHIT allele is absent in >85% of clear cell RCCs (9, 13, 23). To determine whether deletions or rearrangements within the FHIT gene could be detected by Southern blot analysis, we have analyzed DNA from five RCC cell lines and eight primary RCCs with DNA from matched normal kidney tissue. DNAs were digested with two or three restriction enzymes (BamHIII, EcoRV, and/or XbaI), electrophoresed, transferred to a membrane, and hybridized to FHIT cDNA probes, as described previously (18). We detected no FHIT abnormalities by Southern analysis (data not shown), except those reported previously for RC48 and RC49 (18). Additionally, DNAs from 10 RCC cell lines were tested for presence of each FHIT exon by PCR amplification using primer pairs within introns flanking each exon. One RCC cell line, RC48, showed absence of FHIT exons 8–10 by PCR and Southern analysis, and the RC49 cell line showed rearrangement of a FHIT allele, as reported previously. Thus, our analysis of the FHIT gene locus has not shown evidence of frequent alteration.

To assess Fhit transcription in kidney tissues and cell lines, we
have isolated total RNA from three established RCC cell lines, six short-term normal kidney cultures, and eight short-term RCC cultures. Of these, only the RC48 cell line lacked a FHIT RT-PCR product, due to complete absence of exons 8–10. All other RNAs from normal and cancerous cells showed only a normal-sized FHIT RT-PCR product; examples are shown in Fig. 1. Additionally, total RNA was isolated from fragments of tissue from 26 of the tumors tested in Table 1; these samples were also analyzed for FHIT RT-PCR products, and all 26 showed presence of a normal-sized product. Four cases (HT2758, FP2925, ZN2607, and GL2900) showed evidence of an additional aberrant product that lacked exons 4–8 or 4–9. Each of these cases with aberrant RT-PCR products expressed little or no Fhit protein (see below and Table 1).

Because analysis of the structure of the FHIT gene and RT-PCR product detected only a minority of cancer cell lines or tumors with FHIT locus alterations, we considered the possibility that the gene is frequently transcribed at a very low level in kidney cancers. To test this possibility, poly(A)+ RNA from the RCC cell lines CaKil and RC49 was assessed for level of FHIT transcript by Northern blot analysis. Results (Fig. 1, C and D) indicate that in RC49 cancer cells, with an RT-PCR FHIT product that is indistinguishable from normal kidney products (compare Fig. 1A, Lane 8, to other lanes in Fig. 1, A and B). FHIT transcript is nearly undetectable by Northern blot (Fig. 1C, Lane 2), compared to CaKil transcript (Lane 1). We have previously reported that RC49 cells express a very low level of Fhit protein (18). Thus, it could be that RCCs frequently express very little FHIT transcript, due to genetic changes not easily detectable by Southern and PCR analyses.

Fhit Expression. The pattern of Fhit immunoreactivity within the normal kidney was confined to the tubular epithelium and absent in the glomeruli (see Fig. 2A). The protein expression was detected as a red granular chromogen in the cytoplasm of normal renal cells. These results were characteristic for normal kidney control sections. We scored the strongly positive normal controls as (+ + +); the variable chromogen intensity observed in tumor sections was scored as moderately positive (+ +), pale positive (+), or negative (−).

The 41 RCC cases listed in Table 1 were characterized for tumor size, grade, and stage, and sections were assessed for expression of Fhit by standard immunohistochemical methods. Results are summarized in Table 1, and examples of Fhit expression in individual tumor sections are illustrated in Fig. 2, B–F.

Only one tumor, a G3 stage C case ZD2794, was uniformly strongly positive for Fhit expression (Fig. 2, E and F). Five tumors (12%) were pale positive (+) or moderately positive (+ + ), and this category of tumor was about evenly represented in each tumor grade category, three G1, one G2/G3, and one G3. Twenty-one of 41 tumors (51%) were uniformly negative for Fhit expression (see Fig. 2B for example), and 14 of 41 or 34% of tumors showed a mixture of negative and positive tumor cells (see Fig. 2, C and D, for example). Thus, the great majority of clear cell RCCs show reduced or absent Fhit expression.

Correlation of Clinicopathological Parameters with Extent of Fhit Expression. The grading protocol of Fuhrman et al. (25) is based solely on evaluation of nuclear features of the tumor cells and is, thus, simple and reproducible. Briefly, grade 1 (G1) nuclei are round, uniform with inconspicuous nucleoli, whereas G2 and G3 tumors exhibit larger nuclei with irregular outline and prominent nucleoli; G1 tumors are less likely to metastasize than are G2 and G3 tumors. Tumor stage is based on extent of disease spread, with stage A being confined to the kidney, stage B involving perirenal fat invasion, and stage C showing renal vein or vena cava involvement (26, 27). Tumor stage also has prognostic significance.

In summarizing results for our 41 clear cell RCCs for Fhit expression, we have grouped them in Table 1 according to tumor grade, with the first 22 cases judged G1 or mostly G1 (the G1/G2 cases), and of these 22 cases, 17 (77%) are uniformly negative for Fhit expression. This is in clear contrast to the G2 and G3 tumors; of 10 G2 tumors, 2 are negative (20%), and of 9 G3 tumors, 2 are negative (22%). The majority of G2 and G3 tumors exhibit staining patterns of pale positive to moderate positive mixed with a large fraction of Fhit-negative cells and a tendency toward higher fractions of Fhit-negative cells in G3 tumors (data not shown).

The differences in the fractions of Fhit-negative and -positive G1 tumors were compared to the Fhit-negative and -positive fractions in the G2 and G3 grades, and the differences (77% Fhit-negative G1 tumors versus 20% Fhit-negative G2 or 22% Fhit-negative G3 tumors) were significant, as summarized in Table 2. Similarly, 83% of stage A tumors were negative for Fhit expression, whereas 28% of stage B and C tumors were Fhit negative. Most stage B and C tumors exhibited a mixture of Fhit-positive and -negative cells, as summarized in Table 3.

Discussion

Our study of the structure of the FHIT locus in kidney tumors has demonstrated that one FHIT allele is absent in 85–90% of clear cell RCCs (9); a study by Bugert et al. (22) also found absence of one FHIT allele in up to 90% of sporadic nonpapillary RCCs. In our search for alterations in one or both FHIT alleles in RCC cell lines and primary cancers by Southern analysis and by FHIT exon PCR amplification, we have found two cell lines with altered FHIT alleles. van den Berg et al. (20) reported a large homozygous deletion in one of seven RCC cell lines tested by PCR amplification. Very recently, Wang et al. (28) reported homozygous deletions within FHIT intron

Fig. 1. Assessment of FHIT mRNA expression. For RT-PCR analysis, RNAs were reverse-transcribed using a poly(dT) primer and amplified using nested primers as described (20). A, RT-PCR products from lymphoblasts from a t(3;8) carrier (Lane 1); HepG2, a liver cancer cell line (Lane 2); KN5, normal kidney cell short-term culture (Lane 3); KN7, normal kidney cell culture (Lane 4); KN8, normal kidney cell culture (Lane 5); KN9, normal kidney cell (Lane 6); N10, normal kidney cell culture (Lane 7); RC49, RCC cell line (Lane 8); and negative control lane without template (Lane 9). B, RT-PCR products from RC17 RCC cell line (Lane 1); lymphoblastoid line from a t(3;8) carrier (Lane 2); KT3 short-term clear cell RCC culture (Lane 3); and KT6 short-term clear cell RCC culture (Lane 4). For Northern analysis, polyadenylated RNAs were electrophoresed, transferred to membrane, and hybridized to a FHIT cDNA probe representing exons 1–9. C, RNA in Lanes 1 and 2 was from RCC cell lines CaKil and RC49; RNA in Lane 3 was from normal human mammary gland. D, RNAs in C after electrophoresis, illustrating the quantity and quality of the RNAs before transfer to membrane; Lane M, molecular weight marker.
Fig. 2. Immunohistochemical detection of Phit protein in clear cell RCC. A, normal kidney tissue section showing staining for Phit protein (×200). Notice that only tubular epithelium is positive for staining. Two visible glomeruli are clearly negative. B, section from a G1 tumor (case HT2758) showing uniform lack of Phit expression (×200). C, section of tumor SB2549 (G1/G2) with heterogeneously stained cells. Only some cells display strong Phit expression (×200). D, section from case RG3007 (G3 tumor) displaying a heterogeneous pattern of Phit expression with some cells showing Phit protein staining (×200). E, excision specimen of a G3 tumor (case ZD2794) showing cells with strong cytoplasmic staining for Phit protein (×200). F, higher magnification (×400) of the section from the Phit-positive case ZD2794.

4 in eight RCC cell lines; we have tested five of these same RCC cell lines (A704, A498, CaKil, CaKII, and ACHN) for presence of the same fragments by amplification using identical or similar primer pairs and do not confirm homozygous deletions in these RCCs.5 Thus, alteration of both FHIT alleles in RCCs is either infrequent or difficult to detect.

5 T. Druck and K. Huebner, unpublished results.

Assessment of expression of FHIT RNA by the RT-PCR method has also not revealed frequent alterations in RCCs; in addition to the RC48 cell line, which does not express FHIT RNA, the RCC cell line HA335 expresses only aberrant FHIT transcripts (22). Thus, most RCC cell lines and primary tumors express normal FHIT transcripts, although the abundance of these transcripts is not known. Our Northern analysis of FHIT expression suggests that FHIT transcripts are rare in a fraction of RCCs. The immunohistochemical demonstration
of absence of Fhit protein in >50% of the clear cell RCCs tested suggests that both FHIT alleles are inactivated in the majority of clear cell RCCs, although evaluation of the integrity of FHIT RNA and transcripts in RCCs and RCC derived cell lines did not suggest frequent Fhit gene lesions. Although the RT-PCR analysis suggests normal FHIT transcripts in most RCCs, it is possible that, as for the RC49 RCC cells, the transcript is expressed at extremely low levels in RCCs due to unknown mechanisms of inactivation, perhaps involving methylation. The Fhit gene is not imprinted in lymphoblasts or peripheral blood lymphocytes because we have observed expression of both parental alleles. Further detailed studies will be required to define the mechanism of inactivation of Fhit protein in kidney tumors.

This immunohistochemical study of Fhit expression in primary kidney carcinomas has demonstrated that, of the 41 tumors of all grades and stages, 51% were uniformly negative for Fhit expression and another 34% were a mixture of Fhit-positive and -negative cells; thus, 85% of the tumors showed reduced expression of Fhit, a number remarkably close to the 89% of clear cell RCCs for which we found LOH in the t(3;8) region (9). The data strongly suggest that inactivation of the FHIT gene is involved in development of clear cell RCCs and confirm and extend the report of Xiao et al. (29), which showed down-regulation of Fhit expression in RCC cell lines and tumors.

Similarly, >70% of cervical carcinomas showed reduced or absent Fhit expression (30). A recent study of Fhit expression in primary lung carcinomas by immunocytochemistry using the same antiserum in our studies showed absence of Fhit expression in most small cell lung carcinomas and in a large fraction of non-small cell lung carcinomas (31). However, previous studies on Fhit expression have not shown a correlation of lack of Fhit expression with specific clinico-pathological parameters. Our study of clear cell RCCs showed a significant correlation of absence of Fhit expression with low grade (G1) and stage (A), which are themselves favorable prognostic features. On the other hand, our finding of mixed Fhit-positive and -negative cells in G2 and G3 cases suggests that Fhit is being lost in these tumors also, as a secondary genetic event. It would be important to study Fhit expression in metastatic kidney tumors to determine whether secondary Fhit loss is associated with dissemination of these tumors.

The statistically significant correlation of uniform absence of Fhit expression with low-grade and early-stage RCCs suggests the following conclusions: (a) that inactivation of Fhit is an early, possibly initiating, event in development of the majority of grade 1 (G1) tumors; (b) that the G2 and G3 tumors are initiated by inactivation of some other gene on 3p; and (c) that the G2 and G3 tumors secondarily also lose Fhit expression during progression.

These results lend support to the suggestions of earlier studies (15) that Fhit is a tumor suppressor, as indicated by the suppression of tumorogenicity on replacement of Fhit in a kidney carcinoma cell line (24). Additionally, the results show that the familial chromosome translocation break region, the landmark that led to cloning of Fhit, is important in the majority of clear cell RCCs, as expected for a classical tumor suppressor locus.

Table 2 Correlation between tumor grade and Fhit expression

<table>
<thead>
<tr>
<th>Tumor grade</th>
<th>No staining for Fhit</th>
<th>Positive staining for Fhit</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77% (17/22)</td>
<td>23% (5/22)</td>
</tr>
<tr>
<td>Heterogeneous</td>
<td>10% (2/22)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90% (20/22)</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>14% (3/22)</td>
<td>86% (18/22)</td>
</tr>
<tr>
<td>G2</td>
<td>20% (2/10)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80% (8/10)</td>
</tr>
<tr>
<td>Heterogeneous</td>
<td>70% (7/10)</td>
<td>30% (3/10)</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>10% (1/10)</td>
<td>90% (9/10)</td>
</tr>
<tr>
<td>G3</td>
<td>22% (2/9)</td>
<td>78% (7/9)</td>
</tr>
<tr>
<td>Heterogeneous</td>
<td>56% (5/9)</td>
<td>44% (4/9)</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>22% (2/9)</td>
<td>78% (7/9)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The number of G1 tumors expressing Fhit was significantly (P < 0.05) lower compared to G2 and G3 tumors by the Fisher exact test with a two-tailed P.

<sup>b</sup> Both cases had some small areas of cells with G2 features.

<sup>c</sup> Both cases had single cells with positive staining.

Table 3 Correlation between clinical stage and Fhit expression

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>No staining for Fhit</th>
<th>Positive staining for Fhit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83% (15/18)</td>
<td>17% (3/18)</td>
</tr>
<tr>
<td>B-C</td>
<td>26% (6/23)</td>
<td>74% (17/23)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The fraction of Fhit expressing tumors with clinical stage A is significantly (P < 0.05) lower compared to stage B-C tumors by the Fisher exact test with a two-tailed P.

References


Absence or Reduction of Fhit Expression in Most Clear Cell Renal Carcinomas

Piotr Hadaczek, Zurab Siprashvili, Maciej Markiewski, et al.


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