Hypoxia-Inducible Protein 2 (HIG2), a Novel Diagnostic Marker for Renal Cell Carcinoma and Potential Target for Molecular Therapy

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Abstract

To identify molecules to serve as diagnostic markers for renal cell carcinoma (RCC) and as targets for novel therapeutic drugs, we investigated genome-wide expression profiles of RCCs using a cDNA microarray. We subsequently confirmed that hypoxia-inducible protein-2 (HIG2) was expressed exclusively in RCCs and fetal kidney. Induction of HIG2 cDNA into COS7 cells led to secretion of the gene product into culture medium and resulted in enhancement of cell growth. Small interfering RNA effectively inhibited expression of HIG2 in human RCC cells that endogenously expressed high levels of the protein and significantly suppressed cell growth. Moreover, addition of polyclonal anti-HIG2 antibody into culture medium induced apoptosis in RCC-derived cell lines. By binding to an extracellular domain of frizzled homologue 10 (FZD10), HIG2 protein enhanced oncogenic Wnt signaling and its own transcription, suggesting that this product is likely to function as an autocrine growth factor. ELISA analysis of clinical samples identified secretion of HIG2 protein into the plasma of RCC patients even at an early stage of tumor development, whereas it was detected at significantly lower levels in healthy volunteers or patients with chronic glomerulonephritis. The combined evidence suggests that this molecule represents a promising candidate for development of molecular-targeting therapy and could serve as a prominent diagnostic tumor marker for patients with renal carcinomas.

(Cancer Res 2005; 65(11): 4817-26)

Introduction

Renal cell carcinoma (RCC), the third most common malignancy of the genitourinary system, accounts for 2% to 3% of all human malignancies. At present, surgical resection is the most effective treatment for localized RCC tumors, but no satisfactory treatment is available for patients with advanced-stage RCC. Some therapies for RCCs have achieved a response rate of ~20%, but severely adverse reactions are frequent and prognosis for patients does not seem to have improved overall (1, 2). Although tumor stage is considered the most informative prognostic factor, little is known about the underlying molecular mechanisms of renal carcinogenesis.

RCC tumors are characterized based on histologic features as clear cell (80%), papillary (~10%), chromophobe (<5%), or granular, spindle, or cyst-associated carcinomas (5-15%). Each of these histologic subtypes exhibits unique clinical behavior, with clear cell and granular types tending to show more aggressive clinical phenotypes. We chose the most predominant type, clear cell carcinoma, for this study and did a large-scale analysis of gene expression profiles in such tumors. Similar studies reported by other groups have already identified some genes that might be useful for prognostic purposes or for classification of RCCs (3-7).

Here we report identification of a novel RCC-associated molecule, hypoxia-inducible gene 2 (HIG2). The gene encoding HIG2 is one of the transcriptional targets for the activated β-catenin/Tcf-4 complex, and its product functions as an autocrine growth factor that enhances cell growth. These findings have revealed a potential diagnostic marker for early-stage renal tumors and suggest a promising target for development of new drugs for treating RCC.

Materials and Methods

Clinical samples and cell lines. RCC tissues used for cDNA microarray and immunohistochemical analyses were obtained with informed consent from 10 patients who underwent surgical resection at the Kochi Medical School. Clinicopathologic data are summarized in Supplementary Table 1 for each case that was at stage I or II. Clinical stage was judged according to the Unio Internationale Contra Cancrum tumor-node-metastasis classification. Tumor lesions and their surrounding normal tissues were separated, snap-frozen in liquid nitrogen immediately after resection, and stored at ~80°C. At least 90% of the cells in each tumor specimen were identified as tumor cells.

Plasma samples from RCC patients and patients with chronic glomerulonephritis (CGN) used for the ELISA analysis were obtained with informed consent at the Kochi Medical School, Juntendo University School of Medicine, Juntendo University Urayasu Hospital, and Iwate Medical University. In addition, 20 plasma samples from normal healthy volunteers were obtained with informed consent at the Human Genome Center, Institute of Medical Science, The University of Tokyo.

Four cancer cell lines derived from RCC (A498, 786-O, Caki-1, and Caki-2), one from cervical adenocarcinoma (HeLa), nine from colon cancer (SW480, SW948, LoVo, DLD1, HT29, HCT15, HCT116, SNU-C4, and SNU-C5), one from breast cancer (MCF7), four from hepatocellular carcinoma (Huh7, SNU475, HepG2, and Alexander), and one from

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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synovial sarcoma (SYO-1), as well as a line of human embryonic kidney cells (HEK293) and COS7 cells, were grown in monolayers in appropriate media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution. All cultures were maintained at 37°C in air containing 5% CO2.

cDNA microarrays. Fabrication of the cDNA microarray slides has been described elsewhere (8). For analysis of RCC expression profiles and for identifying downstream genes of HIG2 using HIG2 small interfering RNA (siRNA; see Construction of siRNA Expression Vectors), we prepared duplicate sets of slides containing 23,040 or 18,432 cDNA spots, to reduce experimental fluctuation. Briefly, for RCC expression profiles, total RNAs were extracted from RCC patients and from corresponding normal tissues. For identification of downstream genes, total RNAs were extracted 72 hours after transfection from cells containing HIG2 siRNA expression vectors and from cells transfected with psiEGFP vector or mock vector.

T7-based RNA amplification was carried out to obtain adequate quantities of RNA for microarray experiments. Aliquots of amplified RNA were labeled by reverse transcription with adequate amounts of Cy5-dCTP or Cy3-dCTP (Amersham Biosciences, Buckinghamshire, United Kingdom). Hybridization, washing, and detection were carried out as described previously (8). To detect genes that were commonly upregulated in RCC, overall expression patterns of the 23,040 genes on the microarray were first screened to select those with expression ratios of >2.0 that were present in >50% of the RCC cases examined. Finally, to obtain diagnostic markers highly specific to RCC and to identify potential molecular targets, we selected genes that were not expressed in normal kidney, by reference to our own expression database of normal human tissues. To identify the downstream genes of HIG2, we selected genes that were commonly down-regulated (expression ratios of <0.5) in four siRNA-transfected cultures compared with each population of control-transfected cells.

Semiquantitative reverse transcription-PCR analysis. Extracted RNAs were reverse-transcribed using oligo (dT)21 primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the same gene-specific primers as those prepared for constructing our cDNA microarray or with a tubulin-α III (TUBA3) specific primer as an internal control. The primer sequences are listed in Supplementary Table 2. PCR reactions were optimized for the number of cycles to ensure product integrity within the logarithmic phase of amplification.

Production of recombinant HIG2. A cDNA fragment encoding a hydrophilic domain (27-63 amino acid residues) of HIG2 was subcloned into the pET28a vector (Merck, Novagen, Darmstadt, Germany; pET28a-Myc/His) and cloned into the E. coli expression vector psiH1BX-(Supplementary Table 3) into the BbsI restriction enzyme sites of pcDNA3.1 (+)-Myc/His vector (Invitrogen) containing a neo-resistant gene (pcDNA3.1 (+)-Myc/His vector) (Invitrogen) containing a neo-resistant gene (pcDNA3.1 (+)-Myc/His vector). To obtain transfectants that would stably express hHIG2, we transfected pcDNA3.1HIG2 into COS7 cells using FuGene 6 (Roche, Basel, Switzerland), according to the instruction manuals. Transfected cells were cultured in DMEM containing 10% FCS and Geneticin (0.5 mg/mL). Three weeks later, 20 individual colonies were selected and screened for stable transfectants by Limiting Dilution Assay. Each clone was checked for expression of HIG2 by RT-PCR and immunohistochemical staining methods.

Cell growth assays. COS7 cells that stably expressed HIG2 were seeded onto 6-well microtiter plates (1 × 10^4 cells per well) and maintained in medium containing 10% FCS with 0.5 mg/mL of Geneticin for 24, 48, 72, 96, 120 and 144 hours. At each time point, cell numbers were evaluated using Cell Counting Kits (WAKO, Osaka, Japan).

Construction of stable transfectants of hHIG2. The entire coding sequence of human HIG2 (hHIG2) cDNA was amplified by RT-PCR using KOD plus DNA polymerase (TOYOBO, Osaka, Japan) using primers (forward) 5′-TTTCTCTGTG AGAGTAGGG-3′ and (reverse) 5′-CATGCTCTCGTGAGATGG-3′ and cloned into the BamHI restriction enzyme sites of pcDNA3.1 (+)-Myc/His vector (Invitrogen) containing a neo-resistant gene (pcDNA3.1 (+)-Myc/His vector). To obtain transfectants that would stably express hHIG2, we transfected pcDNA3.1HIG2 into COS7 cells using FuGene 6 (Roche, Basel, Switzerland), according to the instruction manuals. Transfected cells were cultured in DMEM containing 10% FCS and Geneticin (0.5 mg/mL). Three weeks later, 20 individual colonies were selected and screened for stable transfectants by Limiting Dilution Assay. Each clone was checked for expression of HIG2 by RT-PCR and immunohistochemical staining methods.

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Effect of RNA interference on cell growth. Each siRNA expression vector was transfected with LipofectAMINE 2000 (Invitrogen) into each of two RCC cell lines (Caki-1 and Caki-2) and a line of human embryonic kidney cells (HEK293), all of which expressed HIG2 endogenously. After 2 weeks of selection by 0.5 mg/mL of Geneticin (Invitrogen), cell numbers were evaluated by Giemsa staining and after 1 week by 3-(4,5-dimethy1thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (10). A knockdown effect of HIG2 mRNA was identified by semiquantitative RT-PCR and Western blot analyses.

Autocrine assay. HIG2-transfected COS7 cells were maintained in FCS-free DMEM for 2 days, then grown with or without HIG2 to confirm autocrine stimulation of cell growth. To confirm an autocrine effect, we added anti-HIG2 pAb, or preimmune rabbit IgG as a negative control, at respective concentrations of 1 μmol/L. Effects of HIG2 and/or its
antibody on cell growth were monitored by counting cells with a hemocytometer. The cells (5 × 10^6 cells per well) were cultured in DMEM containing 0.1% FCS and 100 mmol/L or 1 μmol/L of BSA for 5 days after addition of rhHIG2 recombinant protein. RCC cell lines Caki-1 and Caki-2 were seeded onto 12-well microplates (1 × 10^5 cells per well) and cultured for 5 days in McCoy’s 5A medium containing 0.5% FCS supplemented with 1 μmol/L affinity-purified anti-HIG2 pAb, or with or preimmune rabbit IgG as negative control. Cell numbers were determined as described above.

**Fluorescence-activated cell sorting analysis.** Caki-1 cells were cultured for 5 days in medium containing 0.5 or 1.0 μmol/L of either anti-HIG2 pAb or preimmune rabbit IgG. For fluorescence-activated cell sorting (FACS) analysis, adherent and detached cells were combined and fixed with 70% ethanol at 4°C. After washing with PBS (−) twice, cells were incubated at 37°C for 30 minutes with 1 mL of PBS containing 1 μg of RNase I and stained in 1 mL of PBS containing 50 μg of propidium iodide. The percentages of sub-G1 fractions were determined from at least 2,000 cells in a flow cytometer (FACScalibur; Becton Dickinson, San Diego, CA). To detect interaction between HIG2 and surfaces of Caki-1, Caki-2 or LoVo cells, rhHIG2 and BSA as control were labeled with Alexa Fluor 488, using Alexa Fluor 488 Protein Labeling Kits (Molecular Probes, Inc., Eugene, OR). Cells were exposed to labeled proteins in the culture medium for 1 hour. After two washes with PBS (−), cells were trypsinized and harvested.

**ELISA for HIG2.** To quantify HIG2 protein in plasma of RCC patients, an ELISA was done using affinity-purified anti-HIG2 pAb at a concentration of 10 μg/mL in 50 mmol/L sodium carbonate to coat polystyrene plates (Nalgene Nunc International, Rochester, NY). Excess binding sites were blocked with 2% BSA/PBS at 37°C for 2 hours. Plasma samples from RCC patients, patients with CGN, and normal healthy volunteers were incubated at 37°C for 2 hours. HIG2 protein was detected in each sample by incubating biotinylated anti-HIG2 pAb and peroxidase (HRP)-labeled avidin (Dako Cytomation) with o-phenylenediamine (Dako Cytomation) solution.

**In vitro binding.** One microgram of purified rhHIG2 protein and 150 μg of total cell lysate of COS7 transfected with pcAGGS-HA-FZD10 expression vector or mock vector (pcAGGS-HA) were mixed in 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L CaCl₂, and 1 mmol/L (CH₃COO)₂Mg, respectively, and rotated at 4°C for 4 hours. The complexes were immunoprecipitated using anti-HIG2 pAb and anti-FZD10 pAb, respectively.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) assays were done using ChIP assay Kits (Upstate, Lake Placid, NY) according to the supplier’s manual. Caki-1 and Caki-2 cells were seeded (1 × 10⁶ cells) onto 100-mm culture dishes and maintained for 2 days in serum-free DMEM, with 1 μmol/L rhHIG2 or without rhHIG2. Nuclear extracts were obtained after the histone cross-linking had taken place. DNA bound to immunoprecipitated histone was amplified using primers coding for the 5′-flanking region of HIG2, 5′-ATTCTCCACCTCACAACA-3′ and 5′-TGAGGGAGCTATGCTT-3′.

**Reporter assay.** To examine the activity of the HIG2 promoter, we amplified by PCR four fragments, each corresponding to part of the region flanking HIG2 on the 5′ side, and cloned each product into an appropriate enzyme site of the pGL3-promoter vector (Promega, Madison, WI). Plasmids expressing an activated form of β-catenin (mut β-catenin) and either wild-type or dominant-negative forms of Tcf4 (wtTcf4 and dnTcf4) were prepared as described previously (11). HEK293 cells adjusted to a density of 1 × 10⁵ cells per dish were seeded onto 60-mm dishes. Four micrograms of each reporter plasmid and 4 μg of each construct were cotransfected with 0.45 μg of pRL-TK plasmid (Promega) into cells using FuGene 6 (Roche), to normalize the efficiency of transfection. Reporter assays were carried out using a dual-lucifere reporter assay system according to the supplier’s recommendations (Promega).

**Electrophoretic mobility shift assay.** Gel-shift analysis (electrophoretic mobility shift assay, EMSA) was done as described previously (12). Nuclear extracts were prepared from Caki-1 cells. The probe, a double-stranded 16-nucleotide DNA fragment containing the TCF-binding motif, was prepared by annealing 5′-TAAGTTCACAGGGGT-3′ to 5′-ACCCTTTGAACCTTA-3′ and labeling the product with γ-[32P]ATP using T4 nucleotide kinase. A typical binding reaction contained 10 μg nuclear protein, radiolabeled probe (0.1 ng), and deoxyinosine-deoxyctydine (100 ng, dI-dC) in 25 μL of binding buffer (60 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 10% glycerol). Samples were incubated for 30 minutes at room temperature followed by addition of anti-β-catenin antibody and incubation for another 15 minutes. Electrophoresis was done at 4°C on 4% nondenaturing polyacrylamide (29:1) gels with 0.25× Tris-boric/EDTA buffer. The gels were dried and autoradiographed for 1 hour at −80°C.

**Results**

**Up-regulation of HIG2 in renal cell carcinomas.** In a general, search for novel diagnostic markers for RCC and/or molecular targets for anticancer therapy, we analyzed 10 cases of RCC on a 23,040-cDNA microarray and identified 30 genes (Supplementary Table 4) that were up-regulated in >50% of informative cases. Up-regulation of each gene was confirmed by semiquantitative RT-PCR analysis (data not shown). With a view toward suitability for development of diagnostic markers and/or identification of drug targets, we first selected genes encoding secretory or cytoplasmic membrane proteins, either based on reported information or according to prediction by protein-motif programs SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) or SMART (http://smart.embl-heidelberg.de/). After this selection procedure, we decided to focus on HIG2 (13) because this gene was up-regulated in 9 of the 10 RCCs examined (Fig. 1). Because expression of HIG2 was barely detectable in 20 normal human tissues examined (16 adult and 4 fetal organs), present only in fetal kidney (14), we considered that a drug(s) or antibody targeting this molecule might pose minimal risk of adverse reactions. Expression of HIG2 in tumors developed in other tissues, including 16 cell lines derived from colorectal cancers, breast cancers, and hepatocellular carcinomas was absent or hardly detectable by RT-PCR analysis.

![Figure 1](http://example.com/figure1.png)
indicating exclusive expression in RCCs and fetal kidney (data not shown).

We developed a polyclonal antibody against HIG2 to investigate expression of the protein. Antisera generated in rabbits immunized with rhHIG2 protein were purified using rhHIG2, as described in Experimental Procedures. We first confirmed that the purified HIG2-specific polyclonal antibody (anti-HIG2 pAb) could recognize endogenous HIG2 (7 kDa) protein in RCC cell lines A498, 786-O, Caki-1, and Caki-2 without producing any nonspecific bands, as well as the exogenously introduced myc-tagged HIG2 protein (10 kDa) that was used as positive control (Fig. 2A). Then, to examine extracellular secretion of HIG2, we did immunofluorescent staining using anti-HIG2 pAb and Caki-1 cells. As expected, endogenous HIG2 protein appeared as a granulous pattern in secretion vesicles (Fig. 2B). Furthermore, on Western blots analysis using cell lysates and culture media, HIG2 was detected with anti-HIG2 pAb (Fig. 2C).

**Figure 2.** Subcellular localization of HIG2 protein. A, specificity of purified anti-HIG2 pAb, indicated by expression of exogenous myc-tagged and endogenous HIG2 in COS7 cells and RCC cell lines. The “mock” lane contains lysates of COS7 cells transfected with pcDNA3.1(+) vector; the exogenous myc-tagged HIG2 lane contains lysate of cells transfected with pcDNA3.1(+)–HIG2-myc/His. B, immunofluorescent staining of endogenous HIG2 protein in Caki-1 cells; HIG2 was detected using antirabbit HIG2 pAb and FITC-conjugated antirabbit IgG. C, confirmation of the secretory nature of HIG2. CL, cell lysates. CM, culture media. HIG2 was detected in these preparations using anti-HIG2 pAb. D, immunohistochemical staining of HIG2 protein in sections from normal human adult kidney (i–ii), RCC (iii–iv), and fetal kidney (v–vi) with anti-HIG2 pAb.
lysates and culture medium of COS7 cells that had been transiently transfected with a plasmid designed to express HIG2 (see Experimental Procedures), anti-HIG2 pAb detected secretion of the protein into the culture medium. The amount of secreted protein increased in a time-dependent manner (Fig. 2C). We then did immunohistochemical analysis of HIG2 in surgically resected RCCs and various normal tissues (adult kidney, fetal kidney, heart, liver, lung, pancreas, prostate, and spinal cord). Strong staining was observed only in RCCs (Fig. 2D, iii and iv) and fetal kidney (Fig. 2D, i and ii), but staining in the other tissues was minimal or absent (Fig. 2D, i and ii; Supplementary Fig. L4). We confirmed inhibition of immunohistochemical signals by rhHIG2 (Supplementary Fig. 1B, arrows), a result underscoring the high specificity of our anti-HIG2 pAb. Although we had identified overexpression of HIG2 by analyzing expression profiles of clear cell RCCs only, the immunohistochemical analysis also detected high levels of expression in papillary cell carcinomas but not in RCCs of granular type (Supplementary Fig. 1C).

**Effect of HIG2 on cell growth.** To ascertain a possible role of HIG2 in renal carcinogenesis, we prepared a plasmid designed to express HIG2 (pcDNA3.1 (+)-HIG2-myc/His) and did MTT and colony formation assays. First we transfected the pcDNA3.1 (+)-HIG2-myc/His plasmid into COS7 cells, which express hardly any endogenous HIG2, and established several cell lines that stably overexpressed this gene (data not shown). In a representative line of HIG2-stable cells, growth was significantly enhanced compared with COS7 cells transfected with a control vector (Mock; Fig. 3A). Multiple lines of HIG2-stable cells showed similar HIG2-dependent growth enhancement; the results were confirmed in each line by three independent experiments.

We knocked down expression of endogenous HIG2 in renal cancer cells that abundantly expressed HIG2, using a mammalian vector-based RNAi technique (see Experimental Procedures). Introducing either of two HIG2-specific siRNAs, si#3 and si#4, significantly reduced expression of HIG2 mRNA and protein compared with Mock-siRNA, whereas two others (si#1 and si#2) achieved moderate reduction (Fig. 3B, top). Colony formation and MTT assays revealed that treatment with si#3 or si#4 significantly suppressed growth of Caki-1 and Caki-2 cells, as well as HEK293 (Fig. 3B, bottom and C); si#1 and si#2 caused moderate growth suppression (data not shown). The growth inhibitory effect of the plasmids correlated well with their gene-silencing effects. Taken together, our findings strongly suggested that HIG2 exerts oncogenic activity in RCC cells.

**Autocrine nature of HIG2 growth enhancement.** We prepared a culture medium containing HIG2, derived from the medium used to grow HIG2-overexpressing COS7 cells (see Fig. 3A), and cultured parental COS7 cells in this medium. We also did the same experiment using rhHIG2. Both experiments, whether using native HIG2 or rhHIG2, revealed enhanced growth of the COS7 cells (Fig. 4A and B). The growth-enhancing effect was neutralized by addition of anti-HIG2 pAb but not by preimmune rabbit IgG (Fig. 4A). Furthermore, when we added this antibody to culture medium supporting RCC cell lines Caki-1 and Caki-2, growth of both lines was significantly suppressed as well (Fig. 4C and D).

We then did FACS analysis of Caki-1 cells treated with anti-HIG2 pAb, and observed a dose-dependent increase in the population of apoptotic (sub-G1) cells (Supplementary Fig. 2A), although cancer cell lines derived from other organs, which do not express HIG2, were not influenced by this treatment (Supplementary Fig. 2B). These results strongly support our conclusion that HIG2, a secretory molecule, functions as an autocrine growth factor that is essential for proliferation of renal tumor cells.

**Mechanism of the growth promotion induced by HIG2.** To examine the apparent autocrine oncogenic function of HIG2, we first did cDNA microarray analysis to identify genes whose expression was influenced by the level of HIG2. Each of four HIG2 siRNAs, two of them moderately effective (si#1 and si#2) and two strongly effective (si#3 and si#4), a mock vector, or an EGFp siRNA expression vector, were transfected into HEK293 cells. We used these transfectants to screen genes that were upregulated in clinical RCC samples and commonly down-regulated by reduction of HIG2 expression (Supplementary Table 5). This approach singled out frizzled homologue 10 (FZD10), a G-protein coupled receptor, as a candidate modulator of the growth-signaling activity of HIG2.

To validate our hypothesis that HIG2 might be involved in the Wnt-signaling pathway through FZD10, we did an *in vitro*–binding analysis using recombinant His-tagged HIG2 and anti-FZD10 pAb that was generated using a recombinant protein corresponding to an extracellular domain of FZD10. As shown in Fig. 5A, the experiment revealed that anti-FZD10 pAb and anti-HIG2 pAb precipitated both endogenous FZD10 protein and rhHIG2, respectively. Subsequent FACS analyses revealed that rhHIG2 protein specifically bound to the surfaces of Caki-1 and Caki-2 cells and to synovial sarcoma cells (SYO-1) used as a positive control (15) but not to LoVo cells in which FZD10 expression is barely detectable (Fig. 5B and C). The findings suggested that HIG2 bound to the extracellular domain of FZD10 and that it might therefore exert a regulatory effect on downstream genes related to Wnt-signaling.

To investigate whether HIG2 is self-regulated by the Wnt signaling pathway, we focused on transactivation of the β-catenin/Tcf4 complex, a key player of Wnt signaling. We searched for TCF-binding motifs, 5′-CTTTG A/T A/T-3′ or 5′-A/T A/T CAAAG-3′, within a 3.0-kb genomic DNA fragment of the 5′-region flanking of HIG2, and found two possible candidate sites: one between −2870 and −2864 (TBM1) and the other between −2072 and −2066 (TBM2; Fig. 5D). We then cloned DNA fragments of several lengths corresponding to parts of the 5′-flanking region of HIG2 into an upstream site of the luciferase gene and did a reporter assay using HEK293 cells (Supplementary Fig. 3A). Plasmids pGL3-P1 (containing nucleotides between −2877 and +28) and pGL3-P2 (containing nucleotides between −2090 and +28) revealed 3-fold increases in luciferase activity compared with pGL3 mock vector. Additional vectors containing base substitutions within the binding motif revealed significant reductions in luciferase activity, suggesting that the region between −2072 and −2066 (TBM2) was likely to be the TCF transcriptional target.

To clarify further, we did ChIP assays using RCC cell lines that were stimulated by rhHIG2 and found that β-catenin was bound to the TBM2 site (Supplementary Fig. 3B). Gel-shift assays (EMSA) using nuclear extracts of Caki-1 cells also confirmed specific binding of the complexes (Supplementary Fig. 3C). Moreover, β-catenin was translocated to nuclei when RCC cells were stimulated by addition of rhHIG2 protein in the culture medium; no nuclear staining was observed in nonstimulated cells (Fig. 5E). Cytoplasmic accumulation and nuclear translocation
of β-catenin were observed in a large proportion of clinical RCC (clear cell carcinoma) tissue sections (Fig. 5F). These data suggest that HIG2 stimulates oncogenic Wnt-signaling, and that the gene is activated further by a positive feedback system.

**HIG2 as a diagnostic marker for RCC patients.** To evaluate the applicability of HIG2 as an RCC-specific diagnostic marker, we did sandwich-type ELISA analyses using plasma from 32 RCC patients, as well as plasma from 20 healthy volunteers and 10 patients with CGN to serve as negative controls. We observed much higher levels of HIG2 protein in plasma from each of the 32 RCC patients than was present in either healthy people or CGN patients (Fig. 6A). As expected, HIG2 protein levels in the plasma of two RCC patients with localized tumors (stages I-A and II-A) decreased dramatically after surgical removal of the tumors (Fig. 6B). However, the HIG2 level in plasma from a patient with metastatic RCC (stage IV) was almost unchanged after resection of the primary lesion.

**Discussion**

We have provided substantial evidence here that the product of HIG2, originally identified as a gene induced by hypoxia (13), is likely to function as a crucial growth factor for RCC. Another

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**Figure 3.** Effect of HIG2 on cell growth. A, growth rate of a representative HIG2-stable transformant. HIG2-stable cells revealed enhancement of cell growth compared with mock (vector plasmid only)–transfected cells. Columns, average values from three experiments; bars, ±SD. *P < 0.01, significant differences (Student’s t tests). B, effect of RNAi on growth of RCC cell lines and human embryonic kidney cells. Top, reduction of HIG2 mRNA and protein in RCC cell lines Caki-1 and Caki-2, and cell line HEK293, by siRNA treatment. GAPDH was used as an internal control for RT-PCR, and ACTB served as the control for Western blot analysis. Bottom, colony formation assay demonstrating a decrease in the numbers of colonies after knockdown of HIG2 expression in Caki-1, Caki-2, and HEK293 using an HIG2-specific RNAi, si#4. C, MTT assay showing a decrease in the numbers of viable cells after knockdown of HIG2 expression in the same cell lines using si#4. Columns, average values from five experiments; bars, ±SD. *P < 0.01, significant differences from values derived from a control siRNA, EGFP (estimated by Student’s t tests).
gene, VHL, is often inactivated in sporadic RCCs (16). Mutations or deletions of VHL are responsible for the von Hippel-Lindau disease, causing dysfunction of the ubiquitination machinery and aberrant accumulation of HIF1 protein; accumulated HIF1 evokes the constitutive expression of downstream genes involved in tumor development. However, because we observed remarkably expression of HIG2 in RCC tumors that had shown no abnormalities in VHL (data not shown), up-regulation of HIG2 expression in RCCs seems not a direct effect of VHL mutation or deletion. Using cells established from an HIF1-deficient mouse, Denko et al. (at web site http://171.65.6.67/Hypoxia/outline%20for%20hig2.htm) showed that expression of HIG2 is regulated by HIF1 under hypoxic conditions. HIF1 is composed of two subunits, HIF1α and HIF1β; HIF1α, the oxygen-regulated component that determines HIF1 activity, is rapidly degraded via the ubiquitin-proteasome pathway under normoxic conditions (17). Denko’s group detected expression of HIF1α exclusively in central necrotic areas (a low-oxygen environment in RCC tissue sections). However, we observed abundant HIG2 expression in almost all of the RCC tissue sections regardless to oxidative conditions (Supplementary Fig. 4; Fig. 2D, iii and iv), although expression level of HIG2 was different in each RCC case (Fig. 1) and also the expression levels among cancer cells in each case were heterogenous (Fig. 1). Because there is no possible hypoxia-responsive element (18) within 100 kb upstream of the HIG2 genomic sequence, we suspect that the overexpression of HIG2 in RCCs is largely independent of either VHL or HIF1 regulators.

Microarray analysis revealed that HIG2 was expressed abundantly and exclusively in most of our RCC samples as well as in fetal kidney. We have proposed that HIG2 plays an essential role in proliferation of RCC cells in an autocrine manner, based on the following observations: (i) addition of HIG2 protein to the culture medium enhanced cell growth; (ii) the growth-promoting effect of HIG2 was neutralized by addition of anti-HIG2 antibody to the culture medium; (iii) significant growth suppression of RCC cells occurred when expression of endogenous HIG2 was reduced by HIG2-specific RNAi; and (vi) treatment with anti-HIG2 antibody induced apoptosis of RCC cells. HIG2 protein is probably expressed in precursor cells of the renal tubule of fetal kidney (see Fig. 2B, v and vi).

Figure 4. Detection of an autocrine effect of HIG2 on growth suppression using anti-HIG2 pAb. A, COS7 cultures in medium containing HIG2 showed enhancement of cell growth compared to COS7 cells in medium without HIG2. This effect was neutralized by exposure to anti-HIG2 pAb but not by exposure to preimmune rabbit IgG. Columns, average ratios of absorbance in three experiments; bars, ±SD, compared with samples grown in mock media. *, P < 0.01, significant differences in proliferation rate (Student’s t tests). CM, conditioned media. B, recombinant hHIG2 (rhHIG2) also induced cell outgrowth when total concentrations of BSA and rhHIG2 were adjusted to 1 μmol/L. Columns, average ratios of absorbance from three experiments compared with samples grown in PBS (-) medium; bars, ±SD. * P < 0.01, significant differences in growth rate (Student’s t tests).

C–D, impairment of cancer cell growth after exposure to anti-HIG2 pAb. Cells were exposed for 5 days to preimmune rabbit IgG or anti-HIG2 pAb, at concentrations of 1 μmol/L. Columns, average values from three experiments; bars, ±SD. *, P < 0.01, significant differences in growth rate (Student’s t tests).
of proximal urinary tubules (19), the distribution of HIG2 in fetal kidney suggests that this protein is likely to be involved in early development and organogenesis of renal tissues and also in renal carcinogenesis.

Our experiments also revealed that HIG2 binds to the extracellular domain of FZD10, a Frizzled family cell surface receptor involved in the Wnt signaling pathway. The β-catenin/Tcf4 complex, a key mediator of Wnt-signaling, was shown to

Figure 5. Transcriptional activation by the TCF4/β-catenin complex of downstream signaling pathways of HIG2. A, in vitro–binding analysis of HIG2 and FZD10. B, expression of FZD10 examined in RCC, synovial sarcoma, and colon cancer cell lines. C, interaction of HIG2 and FZD10 expressed on cell surfaces. Cells were incubated with Alexa Fluor 488–labeled BSA and rhHIG2, and interaction was evaluated by FACS. D, schematic representation of various reporter plasmids of HIG2. Putative TCF4-binding motifs are located between −2870 and −2964 bp (TBM1) and between −2072 and −2066 bp (TBM2) from the transcription initiation site. Constructs P1 and P2 contain wild-type binding elements; P1mt and P2mt have 2-nucleotide substitutions in TBM1 and TBM2, respectively. E, translocation of β-catenin in two RCC cell lines after stimulation by rhHIG2 (1 μmol/L). F, immunohistochemical staining of endogenous β-catenin in six RCC tissue specimens. CN, central necrosis. Arrows, staining in nuclei.
bind to the promoter region of the HIG2 gene. Interestingly, cytoplasmic accumulation and nuclear translocation of β-catenin were also identified as a common feature in RCC tissue sections (Fig. 5F). Although mutations of β-catenin are very rare events in RCC, cytoplasmic accumulations of β-catenin have been described previously in renal tumor cells (20–22). A combination of our results with others strongly suggests an important role of stabilized β-catenin protein in renal carcinogenesis. A critical role of the HIG2 autocrine oncogenic pathway as well in development and progression of RCC is indicated by (i) HIG2 activation, (ii) HIG2-FZD10 interaction, (iii) activation of the Wnt signaling pathway, (iv) β-catenin accumulation and stabilization, (v) transcriptional activation of the β-catenin/TCF complex, and (vi) transactivation of HIG2.

From the clinical point of view, markers that are currently available for RCC are not sufficiently sensitive or specific to detect early-stage tumors (23). Some of them cannot even distinguish tumors from inflammatory conditions. Because RCCs at an early stage are amenable to surgical treatment and such patients have markedly better prognoses than those at advanced stages, there is no doubt that development of sensitive diagnostic markers could contribute greatly to improvement of clinical outcomes (23). With that in mind, we developed an ELISA system using anti-HIG2 antibody to measure HIG2 in plasma of RCC patients and were able to detect the protein at much higher levels in plasma of every RCC case examined than in plasma from CGN patients or healthy volunteers. Moreover, the HIG2 levels in plasma of two patients with early-stage RCC were reduced significantly after surgical removal of the tumors, but the level in a patient with metastasis did not change significantly after resection of the primary tumor. Therefore, the assay is specific enough to identify cancer cells, and our combined data strongly imply that HIG2 protein can be a promising candidate as a diagnostic marker for RCCs. However, it is notable that plasma HIG2 levels before surgery did not reflect tumor size or clinical stage. A possible explanation is that larger tumors have poorer blood circulation than smaller tumors and secrete less HIG2 into blood, but additional clinical data will be necessary for addressing that question.

In conclusion, our data have indicated that HIG2 is an essential growth factor for RCC and that a humanized anti-HIG2 monoclonal antibody could be a promising agent for treatment of renal cancer. The exclusive expression of this gene in RCCs and fetal kidney implies that the risk of adverse reactions to antibody therapy should, if present, be minimal. Moreover, an antagonist(s) to the receptor for HIG2 could also be a promising candidate for development of drugs for treatment of RCCs. Finally, our evidence clearly suggests a high likelihood for successful diagnostic application of HIG2. The results documented here should open a new page for diagnosis and treatment of RCC and contribute to a better quality of life for patients with this type of cancer.

Acknowledgments

Received 1/14/2005; revised 3/17/2005; accepted 3/25/2005.

Grant support: Japan Society for the Promotion of Science Research for the Future Program grant #00L01402.

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We thank Kumi Matsuda, Kyoko Kijima, Kie Naito, Yuka Ishizaki, Yumi Kondo, and Yumi Nakajima for technical support; Drs. Chikako Fukukawa, Koichi Okada, Ryo Takata, Ryuichiro Yagyu, Takashi Shimokawa, Satoshi Nagayama, Takahide Arimoto, Toru Nakamura, and Takesufumi Kikuchi for helpful discussions and advice; Dr. Wataru Obara for providing the plasma samples from patients with chronic glomerulonephritis; and Dr. Matsuo Furuhata for advices of immunohistochemical staining in tissue sections.
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